AD_____

Award Number: W81XWH-08-1-0196

TITLE: Jagged-1 signaling pathway in prostate cancer cell growth and angiogenesis

PRINCIPAL INVESTIGATOR: Zhiwei Wang, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
                           Detroit, MI 48201

REPORT DATE: April 2010

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

    ☐X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
April 14 2010	Annual Summary	15 MAR 2008 - 14 MAR 2010

4. TITLE AND SUBTITLE
Jagged-1 signaling pathway in prostate cancer cell growth and angiogenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0196

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Zhiwei Wang, Ph.D.

al5361@wayne.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wayne State University
Department of Pathology
540 East Canfield Avenue
Detroit, MI 48201-1928

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command Fort Detrick Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Prostate cancer is the most commonly diagnosed cancer and is the second leading cause of cancer death in the US. Most patients diagnosed with prostate cancer are treatable, but the patients usually die from hormone refractory (HRPC) and metastatic disease. We have previously shown that expression of Notch receptors and their ligands is upregulated in many cancers including prostate cancer. We hypothesize that inactivation of Jagged-1 signaling, which could be directly due to transcriptional inactivation of Jagged-1 or indirectly due to inactivation of Akt/NF-κB, will not only be a novel approach for the treatment of HRPC and metastases but will also sensitize prostate cancer cells to Taxotere-induced killing. We found that down-regulation of Notch-1 and Jagged-1 induced cell growth inhibition and cell apoptosis. We also found that down-regulation of Notch-1 and Jagged-1 decreased the expression and activities of VEGF, MMP-9, uPA. Moreover, down-regulation of Notch-1 and Jagged-1 inhibited the NF-κB DNA binding activity. Consistent with these results, we also found that the down-regulation of Notch-1 and Jagged-1 by genistein enhanced the antitumor activity of taxotere through Notch/Jagged pathway. Collectively, our results suggest that down-regulation of Jagged-1 and Notch-1 could be useful strategy for treatment of prostate cancer. Based on above hypothesis, this proposal seems highly relevant to the mission of the Department of Defense.

15. SUBJECT TERMS
Notch, Jagged-1, genistein, taxotere, prostate cancer

16. SECURITY CLASSIFICATION OF:

a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
				80	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

Prostate cancer is the most comm only diagnosed cancer and is the second leading cause of cancer death in the US (1). Most patien ts diagnosed with prostate cancer are treatable, but the patients usually die from hormone refractory (HRPC) and metastatic disease. Thus there is a dire need for the developm ent of novel strategies by which HRPC and m etastatic disease could be treated with a bette r outcome. Among many signaling pathways, the Notch signaling pathway has recently emerged and appears to play a critical role in maintaining the balance between cell proliferation, differentiation and apoptosis. There are con siderable evidences sh owing that expression of Notch receptors and their ligands is upregulated in many cancers including prostate cancer (2-7). Hence, perturbation in Notch signaling is believed to contribute to tumorigenesis. It has been reported that Jagged-1 is highly expressed in prostate cancer cells (8, 9). It was found to be increased in the conditioned m edia in prostate cancer cells (10). Jagged-1 is significantly over expressed in m etastatic prostate cancer as co mpared with localized p rostate cancer or ben ign prostatic tissues (11). F urthermore, high Jagged-1 expression in a subset of clinically localized tumors was significan tly associated with recurren ce, independent of other clinical param eters (12). These findings suggest that dysregulation of Ja gged-1 protein levels plays a role in prostate cancer cell growth and progression to metastatic disease. Therefore, down-regulation of Jagged-1 signaling could be a novel approach for the treatment of HRPC and metastatic disease. Data from our laboratory showed that Jagged-1 signaling could be down regulated by Jagged-1 siRNA but more importantly by soy isoflavone genistein (a known chemopreventive agent) and that there is a cross ta lk between Akt/NF-κB and Jagged-1 signaling. We hypot hesize that inactivation of Jagged-1 signaling by genistein, which could be dire ctly due to trans criptional inactivation of Jagged-1 or indirectly due to inactivation of Akt/NF-κB, will not only b e a novel ap proach for the treatment of HRPC and m etastases but will al so sensitize prostate cancer cells to Taxotere-induced killing. The purpose of our cu rrent investigation: is 1) to determine the effect of altered Jagged-1 expression on prostate ca ncer cells. We will determ ine the critica l contribution of Jagged-1 to prostate cancer ce ll proliferation, m igration and invasion by using the Jagged-1-siRNA and Jagged-1-cDNA transf ection experiments for down-re gulation and over-expression of Jagged-1, respectively. 2) to determ ine whether Jagged-1 over-expre ssion contributes to prostate cancer progressi on via activation of Akt/NF- κB pathway. W e will de termine the molecular mechanisms by which Jagged-1 regulates NF- κB and their downstream signaling pathway (VEGF, MMP-2 and MMP- 9), leading to apoptototic cell death and inhibition of invasion and angiogenesis. 3) to test whet her the down regulation of Jagged-1 signaling by chemopreventive agents (geniste in) could sensitize PC-3, D U145, LNCaP and C4-2B prostate cancer cells to Taxotere-induced cell growth i nhibition and apoptosis, and we will also tes t whether the che mo-sensitizing effect of genistei n is m echanistically associated with Jagged-1/NF-κB signaling and its downstream genes, especially MMP-9 and VEGF.

Body of report

Task-1: To determine the effect of altered Jagged-1 expression on prostate cancer cells. We will determine the critical contribution of Jagged-1 to prostate cancer cell proliferation, migration and invasion by using the Jagged-1-siRNA and Jagged-1-cDNA transfection experiments for down-regulation and over-expression of Jagged-1, respectively (Month 1-8):

    a) Conduct our experiments in Jagged-1 siRNA and Jagged-1 cDNA transfected prostate cancer cells to establish the mechanistic role of Jagged-1

    b) The migration and invasion activity of Jagged-1 cDNA or Jagged-1 siRNA transfected cells will be tested

Here it is our observations:

1. Notch signaling pathway in prostate cancer cells. The baseline expression and activation of the Notch signaling mRNA and proteins were determined in a panel of human prostate cancer cell lines that included PC-3, DU145, LNCaP, C4-2B, and MDA PCa2B. The results showed that the Notch signaling pathway was frequently but differentially dysregulated in the different human prostate cancer cell lines (Fig-1). This information will be important in interpreting data on the experiments below.
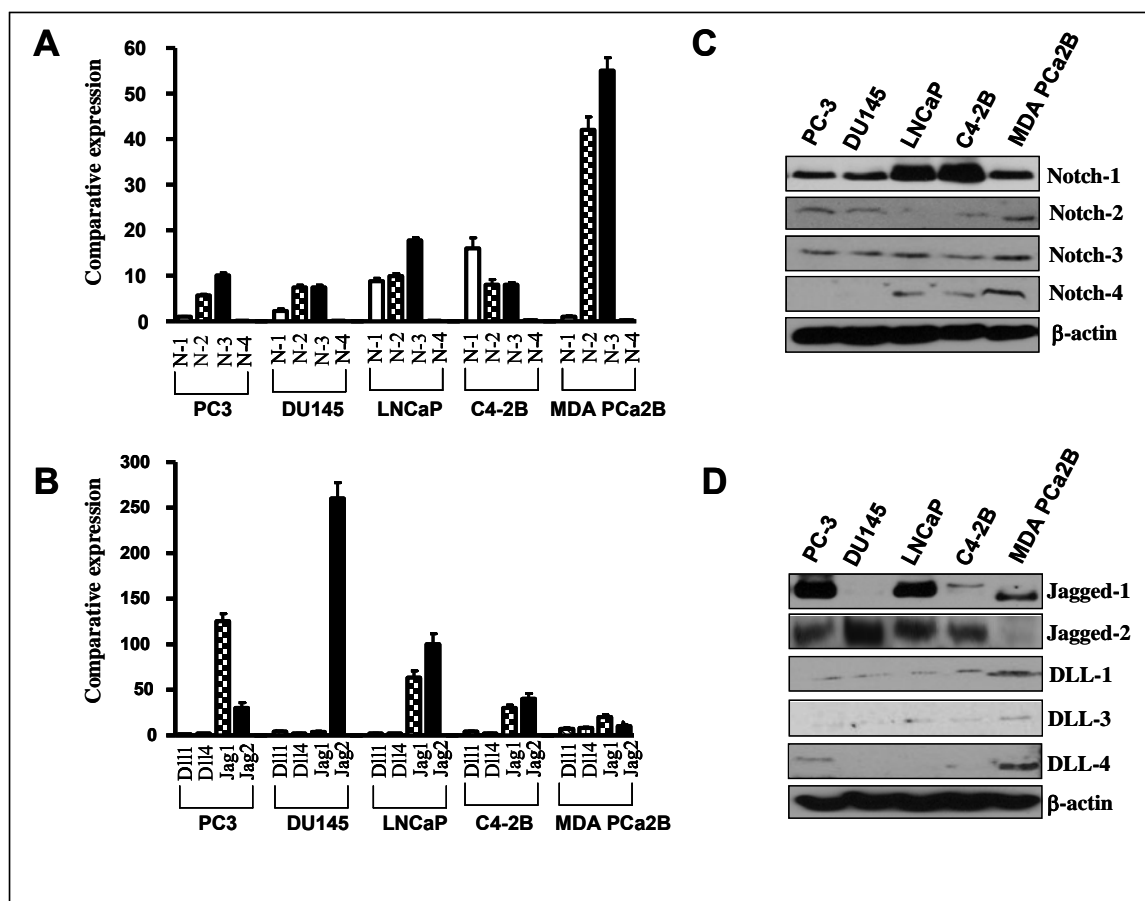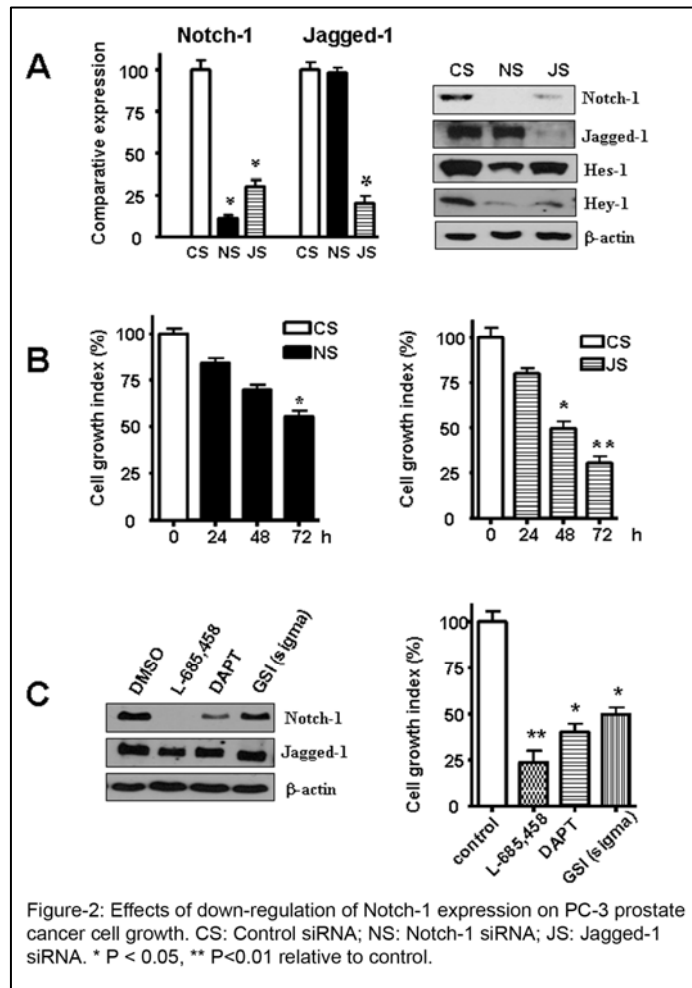

Fig-1: The baseline expression of the Notch signaling mRNA and proteins were determined in a panel of human prostate cancer cell lines. Notch signaling pathway was frequently but differentially dysregulated in the different human prostate cancer cell lines.
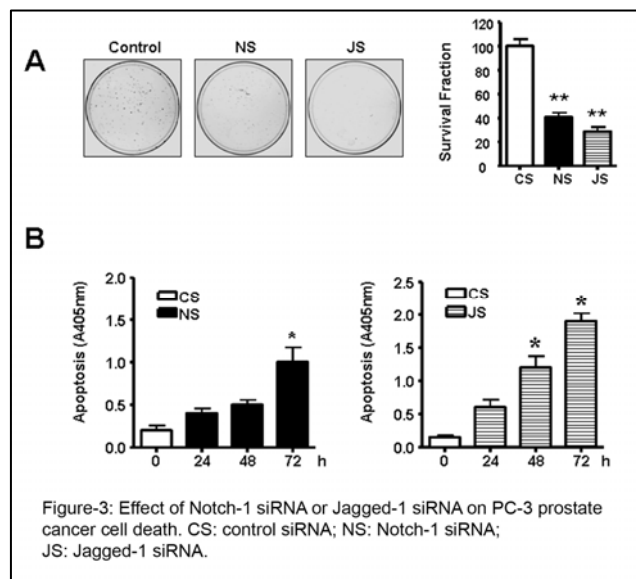
2. Down-regulation of Notch-1 and Jagged-1 expression by siRNA inhibited cell growth and induced apoptosis. We found that both Notch-1 and Jagged-1 at mRNA level and protein levels were barely detectable in Notch-1 siRNA and Jagged-1 siRNA transfected cells, respectively, compared to siRNA control transfected cells (Fig. 2A). We also carried out a standard CBF-1 binding luciferase reporter assay. NICD binds with CBF-1 and other proteins to form a DNA binding complex. As expected, we found that Notch-1 siRNA and Jagged-1 siRNA transfected PC-3 cells co-transfected with the luciferase construct resulted in a significantly decrease in relative luciferase activity, respectively, indicating that the decrease in CBF-1 binding was due to the inhibition of NICD. The cell viability was further determined by MTT assay as shown by Fig. 2B. We found that down-regulation of Notch-1 or Jagged-1 expression by siRNAs caused cell growth inhibition of PC-3 prostate cancer cell line. Moreover, we found that GSI inhibited the Notch-1 expression and consequently GSI had a strong effect in inhibiting the growth of PC-3 cell line (Fig. 2C). In addition, we have also tested the effects of down-regulation of Notch-1 or Jagged-1 on cell viability by clonogenic assay as shown below.

Figure-2: Effects of down-regulation of Notch-1 expression on PC-3 prostate cancer cell growth. CS: Control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. * P < 0.05, ** P<0.01 relative to control.

3. Inhibition of Cell Growth/Survival by Clonogenic Assay. To determine the effect of Notch signaling on cell growth, cells were transfected with Notch-1 siRNA or Jagged-1 siRNA and assessed for cell viability by clonogenic assay. Both Notch-1 siRNA and Jagged-1 siRNA transfection resulted in a significant inhibition of colony formation of PC-3 cells when compared to control (Fig. 3A). Overall, the results from clonogenic assay was consistent with the MTT data as shown in Figure 2B, suggesting that down-regulation of Notch-1 and Jagged-1 inhibited cell growth of PC-3 prostate cancer cells. We investigated whether the overall growth inhibitory

Figure-3: Effect of Notch-1 siRNA or Jagged-1 siRNA on PC-3 prostate cancer cell death. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA.

effects of Notch-1 siRNA or Jagged-1 siRNA are in part due to induction of apoptosis, which was examined by using an ELISA- based assay. These results provided convincing data that down-regulation of Notch-1 or Jagged-1 by siRNAs induced apoptosis in PC-3 prostate cancer cell line (Fig. 3B).

4. Down-regulation of Notch-1 and Jagged-1 decreased PC-3 prostate cancer cell migration and invasion. MMP-9, VEGF and uPA are thought to be critically involved in the processes of tumor cell migration, invasion and metastasis. Because Notch-1 and Jagged-1 siRNA inhibited the expression and activity of MMP-9, VEGF and uPA, we tested the effects of Notch-1 and Jagged-1 down-regulation on cancer cell migration and invasion. We found that down-regulation of Notch-1 and Jagged-1 decreased prostate cancer cell migration. Moreover, as illustrated in Figure 4, Notch-1 and Jagged-1 siRNA transfected cells showed a low level of penetration through the matrigel-coated membrane compared with the control cells. The value of fluorescence from the invaded PC-3 prostate cancer cells was decreased about 3-4 fold compared with that of control cells (Fig. 4).
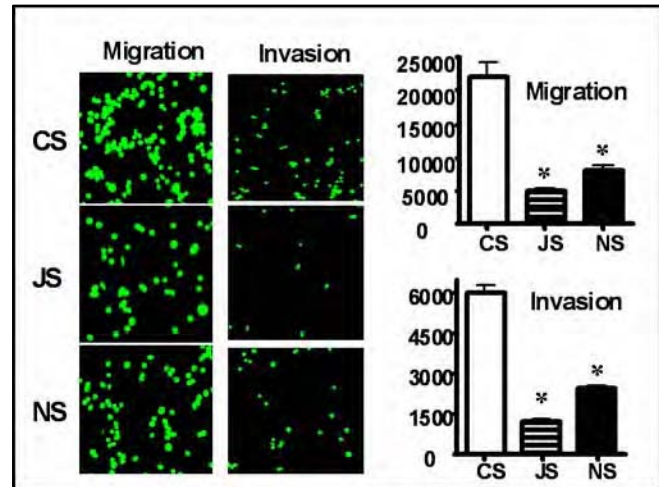

Fig-4: Down-regulation of Notch-1 and Jagged-1 decreased PC-3 prostate cancer cell migration and invasion.

Task-2: To determine whether Jagged-1 over-expression contributes to prostate cancer progression via activation of Akt/NF-κB pathway. We will determine the molecular mechanisms by which Jagged-1 regulates NF-κB and their downstream signaling pathway (VEGF, MMP-2 and MMP-9), leading to apoptototic cell death and inhibition of invasion and angiogenesis (Month 9-16).

   a) Test how Jagged-1 may NF-κB and its downstream genes such as MMP-2, MMP-9 and VEGF.
   b) Test the consequence of MMP-9 and VEGF down regulation on prostate cancer cell angiogenesis.

Here it is our observation:

1. Down-regulation of Notch-1 and Jagged-1 decreased MMP-9 gene transcription and their activities. We therefore investigated whether MMP-9 were down-regulated by Jagged-1 and Notch-1 siRNA in prostate PC-3 cancer cell line. To explore whether Jagged-1 and Notch-1 siRNA transfection could decrease the expression of MMP-9, real-time RT-PCR and Western blotting were conducted. We found that both MMP-9 mRNA and protein levels were dramatically decreased in the Notch-1 and Jagged-1 siRNA transfected cells (Fig. 5). Next, we examined whether the down-regulation of Jagged-1 and Notch-1 could lead to a decrease in MMP-9 activity in prostate cancer cells. There was a marked decrease in the activity of MMP-9 in siRNA transfected cells.

Fig-5: Down-regulation of Notch-1 and Jagged-1 decreased MMP-9 gene transcription, translation and their activities.
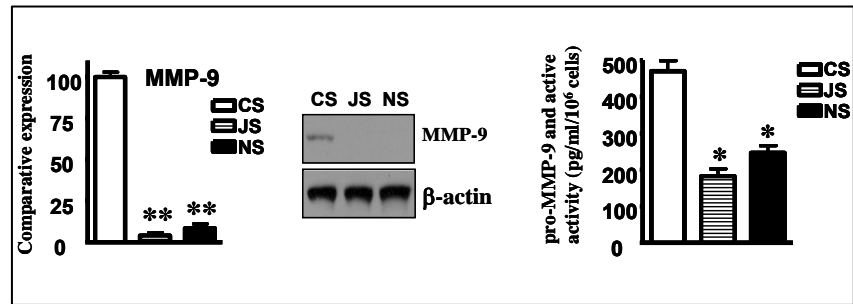
2. Notch-1 siRNA and Jagged-1 siRNA reduced uPA and VEGF gene transcription and translation.

To further investigate whether Notch-1 and Jagged-1 siRNA have any effect on reducing the level of uPA, real-time RT-PCR and Western blotting were done to detect the expression of uPA. We found that both uPA mRNA and protein levels were dramatically reduced in Notch-1 and Jagged-1 siRNA transfected cells (Fig. 6). We also found that the activity of uPA in medium was decreased. Moreover, we found that the expression of VEGF at mRNA and protein levels was decreased by Jagged-1 siRNA or Notch-1 siRNA. To further explore whether Notch-1 siRNA reduced VEGF activity, we examined the levels of
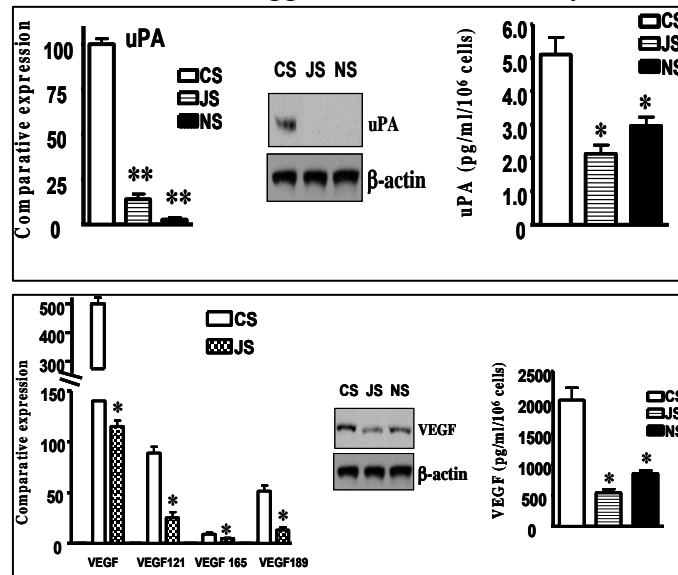
VEGF activity secreted in the culture medium. We found that Notch-1 and Jagged-1 siRNA could lead to a decrease in the levels of VEGF secreted in the culture medium (Fig. 6).

Fig-6: Down-regulation of Notch-1 and Jagged-1 decreased uPA and VEGF gene transcription, translation and their activities.

4. Down-regulation of Notch-1 and Jagged-1 decreased NF-κB DNA binding activity.

Notch signal pathway has been reported to cross-talk with NF-κB signaling pathway. Therefore, we measured the NF-κB DNA-binding activity by EMSA in Notch-1 and Jagged-1 siRNA transfected cells. We found that down-regulation of Notch-1 and Jagged-1 by siRNA transfection decreased NF-κB DNA-binding activity (Fig. 7).
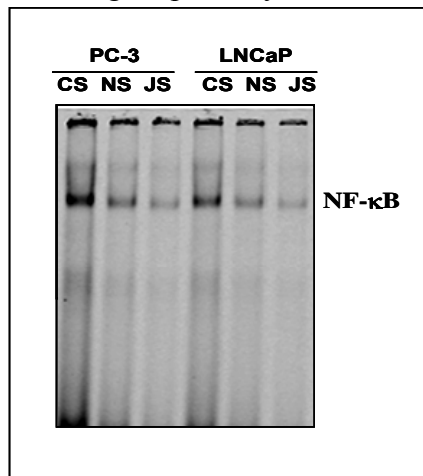

Fig-7: Down-regulation of Notch-1 and Jagged-1 decreased NF-κB DNA binding activity.

8

Task-3: We will test w hether the d own regulation of Jagged-1 sign aling by chemopreventiv e agents (genistein) could sensitize PC-3, DU145, LNCaP and C4-2B prosta te cancer cells to Taxotere-induced cell growth inhibition and apoptosis, and we will also test whether the chemo-sensitizing effect of geni stein is mechanistically associated with Jagged -1/NF-κB signaling and its downstream genes, especially MMP-9 and VEGF (Month 17-24).

a) Conduct our experim ents in Jagged-1 siRNA and Jagged-1 cDNA transfected prostate cancer cells treated with or without genistein to establish the mechanistic role of Jagged-1 during genistein-induced cell growth inhibition and induction of apoptosis.
b) Prostate cancer cells will be exposed to isoflavone for 24 hours followed by treatm ent with Taxotere. We will measure cell growth inhibition by MTT assay and apoptos is by histone/DNA ELISA.
c) Proteins extracted from the cells will be subj ected to Western blot analysis for measuring the levels of Jagged-1 and NF- κB targeted genes (such as Jagged-1, N otch-1, MMP-9, VEGF, COX-2).
d) Measure the DNA binding activity of NF- κB, and all the signals will be quantitated following our standard approach.

Here it is our observations:

1. Genistein induced cell growth inhibition of prostate cancer cells. First, we e xamined the growth inhibitory effects of genistein using the WST assay in four human PCa cell lines such as PC-3, LNCaP, C4-2B, and DU-145. As shown in Fig. 1, the treatment of PCa cells with 15, 30, 50 and 100 µM of genistein resulted in cell growth inhibition in a dose-dependent and tim e-dependent manner in PC-3, LNCaP and C4- 2B cell lines (F ig. 8A). Interestingly, genistein did not significantly inhibit DU-145 cell growth at low concentration. 100 µM genistein caused 80% cell growth inhibition in PC-3, LNCaP and C4-2B, whereas this concentration caused only 40% cell growth inhibition in DU-145. To confirm the results, we have also tested th e effects of treatment on cell viability by clonogenic assay as shown below. To further determ ine the effect of genistein on cell growth, cells were trea ted with genistein and assessed for cell viability by clonogenic assay. Geni stein resulted in a significant inhibition of colony formation of PC-3 and LNCaP cells when com pared with control (Fig. 8B). Overall, th e results from clonogenic assay was consisten t with the WST data, suggesting that genistein inhibits cell growth of PCa cells. Further, to as sess whether the lo ss of cell viability could in part be due to apoptotic cell death, we evaluated the degree of apoptosis induced by genistein using histone-DNA ELISA as shown below. PC-3, LNCaP, C4- 2B and DU-145 cells were treated with 15, 30 and 50 µM genistein for 72 h. After treatm ent, the degree of apoptosis was measured in all four cell lines.
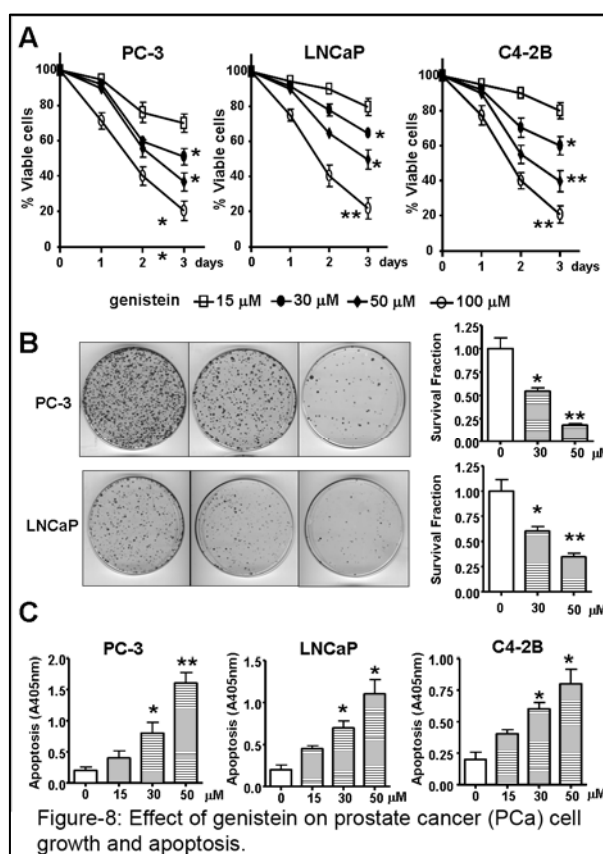

Figure-8: Effect of genistein on prostate cancer (PCa) cell growth and apoptosis.

The induction of apoptosis was found to be do se-dependent in PC-3, LNCaP and C4-2B cel l lines (Fig. 8C). However, geni stein did not induce apoptosis in DU-145 cells. These results provided convincing data showing that genistein could induce apoptosis in PCa cells. In order to further understand the molecular m echanism involved in genistein-induced apoptosis of PCa cells, alterations in the cell survival pathway were investigated. Our previous studies have shown that Notch signaling is over-expressed in PCa and is involved in the inhibition of apoptosis, potentiation of cell growth, and angiogenesis and thus considered as a putative target for drug development. Therefore, we investigated wh ether genistein could regulate Notch signaling pathway.

2. Down-regulation of Notch-1 expression by genistein. Notch-1 mRNA and protein expression in PCa cell line s treated with genistein for 72 h were assessed. We found that Notch-1 was down-regulated by genistein in all thr ee cell lines (Fig. 9A). To confirm the effect on Notch by genistein, we also detected the expression of Notch-1 target gene pAkt in PCa cells after genistein treatment. We found t hat genistein inhibited the pAkt expression (F ig. 9A). Recently, the high expression of F oxM1 was reported in PCa. Down-regulation of FoxM1 by siRNA inhibited cell growth in PCa cell lines. Moreover, FoxM1 has been shown to cross-talk with th e PI3K/Akt pathway. T herefore, we also detected the FoxM1 expression in PCa cells with geniste in treatment. As expected, FoxM1 expression was inhibited by genistein (Fig. 9A). To further confir m our results, we also did imm unofluorescent staining. Indeed, we obs erved lower level of FoxM1 protein in genistein treated cells (Fig. 9B). Next, we assessed whether FoxM1 is downstream of Akt pathway or not for which we designed the experim ents as shown below.
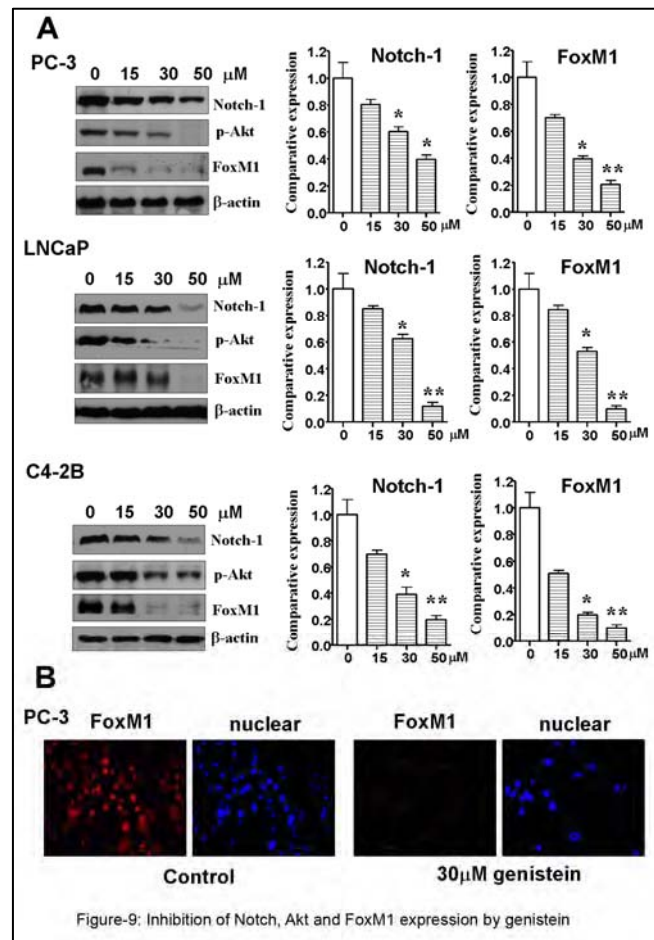

Figure-9: Inhibition of Notch, Akt and FoxM1 expression by genistein

3. Down-regulation of Akt inhibited FoxM1 expression. We detected the basal level of FoxM1 in PC-3, DU145, LNCaP, a nd C4-2B c ell lines using Real-time RT-PCR a nd Western Blotting, respectively. We found t hat FoxM1 is highly expressed in PC-3 and C4-2B, which is consistent with pAkt expression (Fig. 10A). It has been shown that Akt can control FoxM1 expression in osteosarcoma , and thus we sough t to determine whether FoxM1 expression could be controlled by Akt in PCa cells. As expect ed, over-expression of pAkt by Akt cDNA plasm id increased FoxM1 expression in PC-3 cells (Fi g. 10B). However, down-regulation of pAkt by siRNA inhibited FoxM1 expression in PC-3 cells (Fig. 10B). More over, we found that LY294002 and Wortmanin, the PI3K inhibitors, eliminated the expression of FoxM1 (Fig. 10C), suggesting that FoxM1 is regulat ed by Akt pathway in PCa cells . Next, we assessed whether

Notch-1 could regulate the FoxM1 expression b ecause Akt is one of the Notch-1 target genes, which appears to regulate FoxM1.

4. Down-regulation of Notch-1 expression inhibited FoxM1. Down-regulation of Notch-1 by siRNA transfection and gamma secretase inhibitor (GSI, L-685,458, DAPT) treatm ents showed lower expression of Notch-1 prot ein as confirmed by Western blotting (Fig. 11A, 11B). Indeed, inhibition of Notch-1 significantly decreased pA kt and FoxM1 expression in PC-3 and C4-2B cell lines (Fig. 11A, 11B). Conve rsely, over-expression of Notch-1 by ICN transfection showed increased expression of FoxM1 at the mRNA a nd protein levels (Fig. 11B). Furtherm ore, immunofluorescent staining showed higher levels of FoxM1 protein in PC-3 IC N cells (Fig. 11C), suggesting that FoxM1 is regulated by Notch-1.
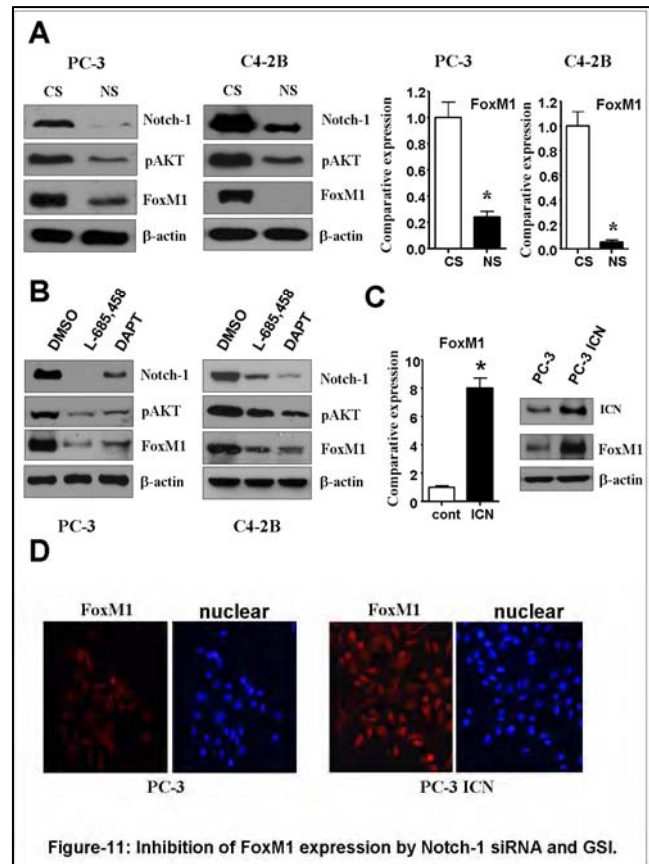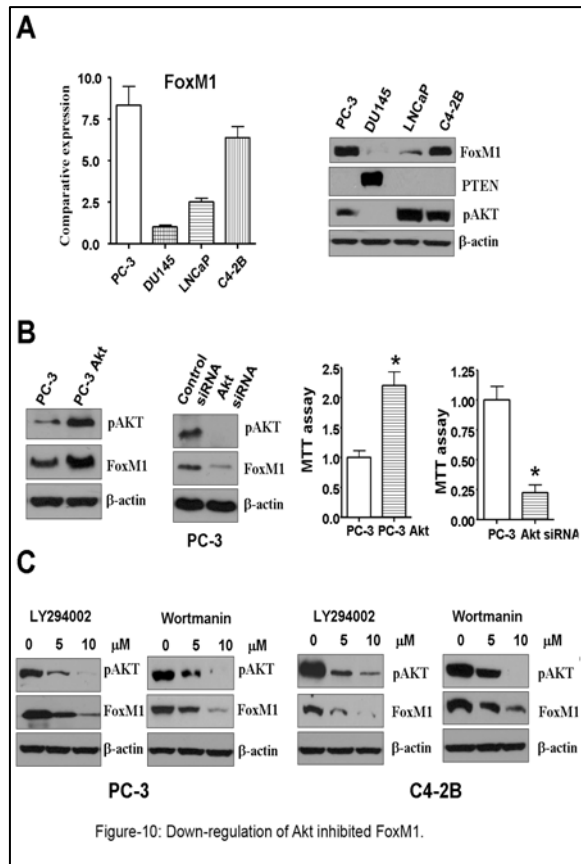
Figure-10: Down-regulation of Akt inhibited FoxM1.

Figure-11: Inhibition of FoxM1 expression by Notch-1 siRNA and GSI.

5. Down-regulation of Notch-1 expression by siRNA potentiates genistein-induced cell growth inhibition and apoptosis. We found that the down-regulat ion of Notch-1 expression significantly inhibited cell growth induced by genistein (Fig. 12). Genistein plus Notch-1 siRNA inhibited cell growth to a greater degree compared to genistein alone. Moreover, Notch-1 siRNA transfected PC-3 cells were si gnificantly more sensitive to s pontaneous and genistein-induced apoptosis (Fig. 12A). However, over-expression of Notch-1 re scued genistein-induced cell growth inhibition and abrogated genistein-induced apoptosis to a certain degree (Fig. 12B). These results provide molecular evidence su ggesting that genistein induced cell growth inhibition and apoptosis is in part mediated through Notch-1 signaling pathway in PCa cells.

6. Genistein-mediated effects on PCa cells were enhanced by taxotere in inhibiting cell growth and causing induction of apoptosis. We found that taxotere did not significantly inhibit

the Notch-1 and pAkt expression (data not shown). However, taxotere inhibited FoxM1 expression in all three PC cells (Fig. 12C). Next, we tested whether genistein could synergize with taxotere leading to enhanced suppression of cell growth as assessed by MTT assay. As can be seen from the results presented in Fig. 12D, 30 μM genistein alone or 1nM taxotere alone caused 40-50% cell growth inhibition, whereas the combination of genistein and taxotere showed drastic inhibition (about 80%) in cell growth, which was also found to be synergistic in inducing apoptotic cell death (Fig. 12D).

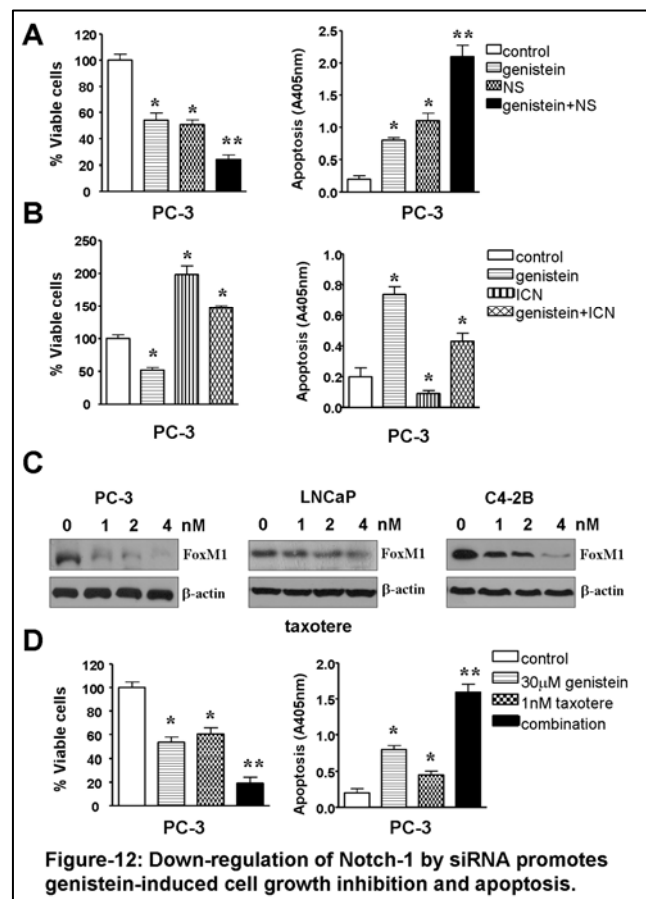These results have been published two papers in "Journal of Cell Biochemistry".

Figure-12: Down-regulation of Notch-1 by siRNA promotes genistein-induced cell growth inhibition and apoptosis.

Key Research Accomplishments

- **We have found that Jagged signaling pathway plays important roles in prostate cancer cells.**

- **Down-regulation of Jagged-1 or Notch-1 induces cell growth inhibition.**

- **Down-regulation of Jagged-1 induced apoptosis in prostate cancer cell lines.**

- **Down-regulation of Notch-1 and Jagged-1 decreased MMP-9 gene transcription and their activities.**

- **Notch-1 siRNA and Jagged-1 siRNA reduced uPA gene transcription and translation.**

- **Notch-1 and Jagged-1 siRNA decreased VEGF activity.**

- **Down-regulation of Notch-1 and Jagged-1 decreased NF-κB DNA binding activity.**

- **Down-regulation of Notch-1 and Jagged-1 decreased PC-3 prostate cancer cell migration and invasion.**

- **Genistein down-regulated the Notch and Jagged-1 expression in prostate cancer cells.**

- **Genistein enhanced the antitumor activity of Taxotere through Notch/Jagged pathway in prostate cancer cells.**

I attended many seminars to learn new technology and new knowledge. I went to AACR meeting every year. After three years train ing under my mentor Dr. Fazlul Sark ar, I will become junior faculty in D epartment of Pathology, Beth Isra el Deaconess Medical Ce nter, Harvard Medical School in May 2011.

Reportable Outcomes
We have published seven papers during the reporting period for this DOD award.

The abstracts of which are presen ted below. Our published review articles also provided comprehensive results that we have obtained.

Abstract: Journal of the cell Biochemistry 2010 109 (4): 726-736
Notch signaling is involved in a variety of cellular processes, such as cell fate specification, differentiation, proliferation, and survival. Notch-1 over-expression has been reported in prostate cancer metastases. Likewise, Notch ligand Jagged-1 was found to be over-expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues, suggesting the biological significance of Notch signaling in prostate cancer progression. However, the mechanistic role of Notch signaling and the consequence of its down-regulation in prostate cancer have not been fully elucidated. Using multiple cellular and molecular approaches such as MTT assay, apoptosis assay, gene transfection, real-time RT-PCR, Western blotting, migration, invasion assay and ELISA, we found that down-regulation of Notch-1 or Jagged-1 was mechanistically associated with inhibition of cell growth, migration, invasion and induction of apoptosis in prostate cancer cells, which was mediated via inactivation of Akt, mTOR and NF-κB signaling. Consistent with these results, we found that the down-regulation of Notch-1 or Jagged-1 led to decreased expression and the activity of NF-κB downstream genes such as MMP-9, VEGF and uPA, contributing to the inhibition of cell migration and invasion. Taken together, we conclude that the down-regulation of Notch-1 or Jagged-1 mediated inhibition of cell growth, migration and invasion, and the induction of apoptosis was in part due to inactivation of Akt, mTOR and NF-κB signaling pathways. Our results further suggest that inactivation of Notch signaling pathways by innovative strategies could be a potential targeted approach for the treatment of metastatic prostate cancer.

Abstract: Journal of the cell Biochemistry 2011 112 (1): 78-88
Genistein is a known inhibitor of protein tyrosine kinases, which is known to inhibit cell proliferation and induces apoptotic cell death. Although many studies have been done to uncover the mechanisms by which genistein exerts its antitumor activity, the precise molecular mechanisms remain to be unclear. In the present study, we assessed the effects of genistein on cell viability and apoptosis in prostate cancer. For mechanistic studies, we used multiple cellular and molecular approaches such as gene transfection, real-time RT-PCR, Western blotting, invasion assay and ELISA. For the first time, we found a significant reduction in cell viability in genistein treated cells, which was consistent with induction of apoptosis and also associated with

down-regulation of Notch-1, Akt and FoxM1. We also found down-regulation of FoxM1 by Taxotere in prostate cancer cells. Moreover, we found that genistein enhanced the antitumor activity of Taxotere in prostate cancer cells. Taken together, we conclude that the down-regulation of Notch-1 by genistein could be an effective approach, which will cause down-regulation of Akt and FoxM1, resulting in the inhibition of cell growth. These results suggest that antitumor activity of genistein is mediated through a novel mechanism involving inactivation of Notch-1/Akt/FoxM1 signaling pathways.

Abstract: Biochim Biophys Acta reviews on cancer 2010 1806 (2): 258-67
Chemotherapy is an important therapeutic strategy for cancer treatment and remains the mainstay for the management of human malignancies; however, chemotherapy fails to eliminate all tumor cells because of intrinsic or acquired drug- resistance, which is the most common cause of tumor recurrence. Recently, emerging evidences suggest that Notch signaling pathway is one of the most important signaling pathways in drug-resistant tumor cells. Moreover, down-regulation of Notch pathway could induce drug sensitivity, leading to increased inhibition of cancer cell growth, invasion, and metastasis. This article will provide a brief overview of the published evidences in support of the roles of Notch in drug-resistance, and will further summarize how targeting Notch by "natural agents" could become a novel and safer approach for the improvement of tumor treatment by overcoming drug-resistance.

Abstract: Biochim Biophys Acta reviews on cancer 2010 1806 (2): 122-30
Platelet-derived growth factor-D (PDGF-D) can regulate many cellular processes, including cell proliferation, apoptosis, transformation, migration, invasion, angiogenesis and metastasis. Therefore PDGF-D signaling has been considered to be important in human malignancies, and thus PDGF-D signaling may represent a novel therapeutic target, and as such suggests that the development of agents that will target PDGF-D signaling is likely to have a significant therapeutic impact on human cancers. This mini-review describes the mechanisms of signal transduction associated with PDGF-D signaling to support the role of PDGF-D in the carcinogenesis. Moreover, we summarize data on several PDGF-D inhibitors especially naturally occurring "chemopreventive agent" such an indole compound, which we believe could serve as a novel agent for the prevention of tumor progression and/or treatment of human malignancies by targeted inactivation of PDGF-D signaling.

Abstract: Cancer Letters 2010 292 (2): 141-8
Notch signaling pathways are known to regulate many cellular processes, including cell proliferation, apoptosis, migration, invasion, and angiogenesis, and is one of the most important signaling pathway during normal development. Recently, emerging evidences suggest that microRNAs (miRNAs) can function as key regulators of various biological and pathologic processes during tumor development and progression. Notch signaling has also been reported to be regulated through crosstalk with many pathways and factors where miRNAs appears to play a major role. This article will provide a brief overview of the published evidences for the crosstalks between Notch and miRNAs. Further, we summarize how targeting miRNAs by natural agents could become a novel and safer approach for the prevention of tumor progression and treatment.

Abstract: Cancer Letters 2010 279 (1): 8-12

The Notch signaling pathway is known to be responsible for maintaining a balance between cell proliferation and death and, as such, plays important roles in the formation of many types of human tumors. Recently, Notch signaling pathway has been shown to control stem cell self renewal and multi-potency. As many cancers are thought to be developed from a number of cancer stem-like cells, which are also known to be linked with the acquisition of epithelial-mesenchymal transition (EMT); and thus suggesting an expanding role of Notch signaling in human tumor progression.

Abstract: Anticancer Research 2010 279 (1): 8-12
The Notch signaling pathway appears to be responsible for maintaining a balance between cell proliferation and apoptosis and thus it has been suggested that Notch may play an important role in species development and in the development and progression of several malignancies. Therefore, the Notch signaling pathway may represent a novel therapeutic target, which could have the highest therapeutic impact in modern medicine. This review describes the mechanisms of signal transduction of the Notch signaling pathway and provides emerging evidence in support of its role in the development of human malignancies. Further attempts have been made to summarize the role of several chemopreventive agents that could be useful for targeted inactivation of Notch signaling, which could become a novel approach for cancer prevention and treatment.

<u>**Conclusion:**</u> The all experiments have been completed. We have published seven papers. We have found and reported that Jagged-1 and Notch play important roles in prostate cancer cell proliferation, migration and invasion via activation of Akt/NF-κB pathway and their downstream genes such as MMP-9, VEGF, leading to apoptototic cell death and inhibition of invasion and angiogenesis. Moreover, down regulation of Jagged-1 signaling by chemopreventive agents (genistein) could sensitize prostate cancer cells to Taxotere-induced cell growth inhibition and apoptosis, which is mechanistically associated with Jagged-1/N F-κB signaling and its downstream genes, especially MMP -9 and VEGF. These studies provided the mechanisms to target Jagged-1/Notch pathway for cancer therapy.

Reference List

1 Jemal,A., Siegel,R., Ward,E., Hao,Y., Xu,J. and Thun,M.J. Cancer statistics, 2009, CA Cancer J.Clin., *59:* 225-249, 2009.

2 Miele,L. and Osborne,B. Arbiter of differentiation and death: Notch signaling meets apoptosis, J.Cell Physiol, *181:* 393-409, 1999.

3 Miele,L., Miao,H. and Nickoloff,B.J. Notch signaling as a novel cancer therapeutic target, Curr.Cancer Drug Targets., *6:* 313-323, 2006.

4   Wang,Z., Zhang,Y., Banerjee,S., Li,Y. and Sarkar,F.H. Inhibition of nuclear factor kappab activity by genistein is mediated via Notch-1 signaling pathway in pancreatic cancer cells, Int.J.Cancer, *118:* 1930-1936, 2006.

5   Wang,Z., Li,Y., Banerjee,S. and Sarkar,F.H. Exploitation of the Notch signaling pathway as a novel target for cancer therapy, Anticancer Res., *28:* 3621-3630, 2008.

6   Wang,Z., Li,Y., Banerjee,S. and Sarkar,F.H. Emerging role of Notch in stem cells and cancer, Cancer Lett., *279:* 8-12, 2009.

7   Wang,Z., Li,Y., Kong,D., Ahmad,A., Banerjee,S. and Sarkar,F.H. Cross-talk between miRNA and Notch signaling pathways in tumor development and progression, Cancer Lett., 2009.

8   Dalrymple,S., Antony,L., Xu,Y., Uzgare,A.R., Arnold,J.T., Savaugeot,J., Sokoll,L.J., De Marzo,A.M. and Isaacs,J.T. Role of notch-1 and E-cadherin in the differential response to calcium in culturing normal versus malignant prostate cells, Cancer Res., *65:* 9269-9279, 2005.

9   Velasco,A.M., Gillis,K.A., Li,Y., Brown,E.L., Sadler,T.M., Achilleos,M., Greenberger,L.M., Frost,P., Bai,W. and Zhang,Y. Identification and validation of novel androgen-regulated genes in prostate cancer, Endocrinology, *145:* 3913-3924, 2004.

10 Martin,D.B., Gifford,D.R., Wright,M.E., Keller,A., Yi,E., Goodlett,D.R., Aebersold,R. and Nelson,P.S. Quantitative proteomic analysis of proteins released by neoplastic prostate epithelium, Cancer Res., *64:* 347-355, 2004.

11 Santagata,S., Demichelis,F., Riva,A., Varambally,S., Hofer,M.D., Kutok,J.L., Kim,R., Tang,J., Montie,J.E., Chinnaiyan,A.M., Rubin,M.A. and Aster,J.C. JAGGED1 expression is associated with prostate cancer metastasis and recurrence, Cancer Res., *64:* 6854-6857, 2004.

12 Santagata,S., Demichelis,F., Riva,A., Varambally,S., Hofer,M.D., Kutok,J.L., Kim,R., Tang,J., Montie,J.E., Chinnaiyan,A.M., Rubin,M.A. and Aster,J.C. JAGGED1 expression is associated with prostate cancer metastasis and recurrence, Cancer Res., *64:* 6854-6857, 2004.

Down-Regulation of Notch-1 Is Associated With Akt and FoxM1 in Inducing Cell Growth Inhibition and Apoptosis in Prostate Cancer Cells

Zhiwei Wang,[1*] Yiwei Li,[1] Aamir Ahmad,[1] Sanjeev Banerjee,[1] Asfar S. Azmi,[1] Dejuan Kong,[1] Christine Wojewoda,[1] Lucio Miele,[2] and Fazlul H. Sarkar[1*]

[1]*Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan*
[2]*University of Mississippi Cancer Institute, Jackson, Mississippi*

ABSTRACT

Although many studies have been done to uncover the mechanisms by which down-regulation of Notch-1 exerts its anti-tumor activity against a variety of human malignancies, the precise molecular mechanisms remain unclear. In the present study, we investigated the cellular consequence of Notch-1 down-regulation and also assessed the molecular consequence of Notch-1-mediated alterations of its downstream targets on cell viability and apoptosis in prostate cancer (PCa) cells. We found that the down-regulation of Notch-1 led to the inhibition of cell growth and induction of apoptosis, which was mechanistically linked with down-regulation of Akt and FoxM1, suggesting for the first time that Akt and FoxM1 are downstream targets of Notch-1 signaling. Moreover, we found that a "natural agent" (genistein) originally discovered from soybean could cause significant reduction in cell viability and induced apoptosis of PCa cells, which was consistent with down-regulation of Notch-1, Akt, and FoxM1. These results suggest that down-regulation of Notch-1 by novel agents could become a newer approach for the prevention of tumor progression and/or treatment, which is likely to be mediated via inactivation of Akt and FoxM1 signaling pathways in PCa. J. Cell. Biochem. 112: 78–88, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: NOTCH-1; PROSTATE CANCER; CELL GROWTH; APOPTOSIS; Akt; FoxM1

Although prostate cancer (PCa) mortality has been decreased in recent years, it is still the second leading cause of cancer-related deaths in men in the United States [Jemal et al., 2009]. Therefore, there is a tremendous need for the development of mechanism-based strategies by which PCa could be treated with a better outcome. Notch signaling has been very attractive due to its functions in a variety of cellular processes, including differentiation, proliferation, and survival [Rizzo et al., 2008]. Four Notch receptors (Notch 1–4) and five ligands (Jagged-1, 2, Delta-1, 3, 4) have been described in mammals [Miele et al., 2006]. Binding of ligand to its receptor induces metalloproteinase-mediated and gamma secretase-mediated cleavage of the Notch receptor. The Notch intracellular domain (ICN) is released from the plasma membrane and translocates into the nucleus and activates its target genes [Miele, 2006; Wang et al., 2008]. Notch signaling pathway was found to be over-expressed in PCa cell lines [Shou et al., 2001; Wang et al., 2010b]. Moreover, Notch signaling pathways play important roles in

prostate development and progression [Leong and Gao, 2008; Bin et al., 2009].

Recently, another signaling pathway, namely FoxM1, has been shown to be over-expressed in PCa and studies have shown that alterations in FoxM1 signaling were associated with carcinogenesis [Kalin et al., 2006; Chandran et al., 2007; Pandit and Gartel, 2010]. Specifically, FoxM1 signaling network is frequently up-regulated in most human malignancies including lung cancer, glioblastomas, PCa, basal cell carcinomas, hepatocellular carcinoma, breast cancer, and pancreatic cancer [Gartel, 2008, 2010; Wang et al., 2010a], suggesting that FoxM1 is a major player in human cancers. Moreover, it has been shown that higher expression of FoxM1 was associated with poor prognosis in breast cancer and gastric cancer patients [Bektas et al., 2008; Li et al., 2009]. These results suggest that FoxM1 may have a critical role in the development and progression of human cancers especially PCa. Therefore, it is believed that inactivation of FoxM1 could represent a promising

strategy for the development of novel and selective anti-cancer therapies. It has been shown that Akt is Notch downstream gene [Wang et al., 2010b] and Akt can control FoxM1 expression in osteosarcoma [Major et al., 2004], and thus we sought to determine whether FoxM1 expression could be controlled by Notch and Akt in PCa cells in the present study. Although several chemical agents such as gamma secretase inhibitors, siomycin A, and thiostrepton have been shown to inhibit Notch and FoxM1 activity, respectively, they also demonstrated unwanted toxicity in mice and human. Therefore, we also investigated whether a non-toxic "natural agent" could be useful for the inhibition of Notch signaling which consequently may also inactivate Akt and FoxM1 signaling, and thus it could be beneficial for the prevention of tumor progression and/or therapy for PCa.

Taxotere (Docetaxel) has shown clinical activity in a wide spectrum of solid tumors including PCa [Chiuri et al., 2009]. Taxotere has been reported to inhibit cell growth and induce apoptosis in PCa [Li et al., 2005a,b,c]. Clinical trials have shown that the combination chemotherapy using taxotere with other agents improves survival in PCa patients [Falci et al., 2009]. However, the combination treatment contributes to a certain degree of dose-related toxicity. Therefore, there is a dire need for the development of therapeutic strategies to improve efficacy and reduce side effects of taxotere-based treatment. Naturally occurring agents such as genistein is a prominent isoflavone found in soybeans, has been found to inhibit cell growth and induce apoptosis in vitro and in vivo without toxicity [Banerjee et al., 2008]. Studies from our laboratory have also found that genistein can inhibit NF-κB and Akt activation in PCa cells, suggesting its anti-tumor activity against PCa [Banerjee et al., 2005, 2007b; Li et al., 2005a]. It has been reported that NF-κB is regulated by Notch signaling in human cancer [Wang et al., 2006a, 2008; Osipo et al., 2008], which became the basis for conducting the current study to test whether genistein could inhibit the Notch signaling in PCa cells and how it is related to other signaling pathways.

In the present study, we sought to gain molecular evidence in support of the mechanistic consequence of Notch-1 down-regulation in cell growth and apoptosis using human PCa cells. Our results show that down-regulation of Notch-1 could be an effective approach for inhibiting cell growth and inducing apoptotic cell death, which was mechanistically associated with inactivation of Akt and FoxM1. Moreover, we found that genistein could inhibit cell growth and also could induce apoptotic cell death in PCa, which appears to be in part mediated via inactivation of Notch-1/Akt/FoxM1 signaling pathways. Together, we believe that inactivation of Notch-1 by novel non-toxic agents could be a potential targeted approach for the prevention of tumor progression and/or treatment of human PCa, which we believe would be due to inactivation of Notch-1 downstream genes such as Akt and FoxM1.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL REAGENTS
Human PCa cell lines including PC-3, DU145, LNCaP, and C4-2B were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA)

supplemented with 10% FBS. The cell lines have been tested and authenticated through our Genomic Core Facility, Applied Genomics Technology Center at Wayne State University. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). Experimental reagents were described in Supplementary Methods.

PLASMIDS AND TRANSFECTION STUDIES
The Notch-1 ICN cDNA plasmid encoding the Notch-1 intracellular domain was described as before [Weijzen et al., 2002]. PCa cells were transfected with Notch-1 siRNA and siRNA control, respectively, using Lipofectamine 2000.

REAL–TIME REVERSE TRANSCRIPTION–PCR ANALYSIS FOR GENE EXPRESSION STUDIES
The total RNA from treated and untreated cells was isolated by Trizol (Invitrogen) and purified by RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's protocols. The primers used in the PCR reaction and real-time PCR amplifications were performed as described earlier [Wang et al., 2006a, 2007].

WESTERN BLOT ANALYSIS
Cells were lysed in lysis buffer by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS–PAGE and transferred onto a nitrocellulose membrane for Western blotting as described before [Wang et al., 2006a].

CLONOGENIC ASSAY
To test the survival of cells treated with genistein, PCa cells were plated (50,000–100,000 per well) in a six-well plate and incubated overnight at 37°C. After 72 h exposure to various concentrations of genistein, the cells were subjected to clonogenic assay as described earlier [Wang et al., 2009]. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS
The Cell Death Detection ELISA Kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, treated cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis as described earlier [Wang et al., 2006b].

FLOW CYTOMETRY AND CELL–CYCLE ANALYSIS
The cells were synchronized in G_0 by serum starvation for 24 h in phenol red-free RPMI with 0.1% serum. Subsequently, cells were released into complete media containing 10% FBS. The cell cycle was analyzed by flow cytometry as described earlier [Wang et al., 2006b].

CELL GROWTH INHIBITION STUDIES BY MTT ASSAY
The PCa cells (5×10^3) were seeded in a 96-well culture plate and subsequently treated with taxotere and incubated with MTT reagent

(0.5 mg/ml) at 37°C for 2 h and MTT assay was performed as described earlier [Wang et al., 2006b].

IMMUNOFLUORESCENCE MICROSCOPY
Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in PBS and 10% goat serum blocking solution for 1 h. The cells were incubated for 2 h with anti-FoxM1 in 5% goat serum and were stained, and viewed as described earlier [Wang et al., 2010b].

MMP-9 AND VEGF ACTIVITY ASSAY
The cells were seeded in six-well plate and incubated at 37°C. After 24 h, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 72 h. MMP-9 and VEGF activity in the medium was detected as described before [Wang et al., 2010b].

CELL INVASION ASSAY
The invasive activity of the cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) as described earlier [Wang et al., 2006a].

ANIMAL EXPERIMENTS
The severe combined immunodeficiency (SCID)-human model of experimental PCa was used for our study as described earlier [Banerjee et al., 2007a]. Briefly, suspensions of PC-3 and C4-2B cells were injected, respectively, by insertion of a 27-gauge needle through the mouse (Taconic Farms) skin directly into the marrow surface of the previously implanted bone. The mice were divided into two groups of seven animals in each group. In the genistein treatment groups, the mice were fed a genistein containing diet (1 g/kg diet) beginning on the 30th day after intraosseous PC-3 cell injection as described before [Li et al., 2006]. The C4-2B xenograft mice were treated with 7.5 mg genistein/100 μl/mice by gavage every day for 4 weeks beginning on the 30th day after intraosseous C4-2B cell injection. All mice were sacrificed on the 28th day after genistein treatment because big tumors were formed in control mice. H&E staining confirmed the presence of tumor.

DENSITOMETRIC AND STATISTICAL ANALYSIS
The statistical significance of differential findings between experimental groups and control was statistically evaluated using GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). P-values lower than 0.05 were considered statistically significant.

RESULTS

NOTCH SIGNALING PATHWAY IN PROSTATE CANCER CELLS
First, the baseline expression of Notch signaling molecules was determined using real-time RT-PCR and Western blotting analysis, respectively, in a panel of human PCa cell lines that included PC-3, DU145, LNCaP, and C4-2B. The results showed that the Notch signaling pathway was frequently but differentially dysregulated in different human PCa cell lines (Fig. 1). It is important to note that we focused our studies on the cleaved Notch because it is the active

functional form of Notch. Therefore, Notch in our all figure legends means active cleaved Notch. We found that Notch-1 was highly expressed in PC-3, LNCaP, and C4-2B. Previous studies have shown that down-regulation of Notch-1 inhibited cell growth, induced apoptosis, and decreased cell invasion of PCa cells [Zhang et al., 2006; Wang et al., 2010b]. In an effort to confirm our results, and to further investigate the precise molecular roles of Notch-1 in PCa cells, we have used stable clones (name PC-3 ICN) of PC-3 cells transfected with ICN plasmid. We found that the over-expression of Notch-1 promoted cell growth, inhibited apoptosis, increased S-phase fractions, and increased cell invasion, all of which were associated with up-regulation of pAkt, NF-κB, and its target gene VEGF and MMP-9 (Supplementary Figs. 1 and 2), which provided direct molecular evidence in support of the role of Notch-1 in tumor aggressiveness.

DOWN-REGULATION OF Akt INHIBITED FoxM1 EXPRESSION
Recently, high expression of FoxM1 was reported in PCa. Down-regulation of FoxM1 by siRNA inhibited cell growth in PCa cell lines [Kalin et al., 2006]. Moreover, FoxM1 has been shown to cross-talk with the PI3K/Akt pathway [Major et al., 2004; Wang et al., 2010a,b]. Therefore, we assessed whether FoxM1 is downstream of Akt pathway or not, and for which we designed the experiments as shown below. First, we detected the basal level of FoxM1 in PC-3, DU145, LNCaP, and C4-2B cell lines using real-time RT-PCR and Western blotting, respectively. We found that FoxM1 is highly expressed in PC-3 and C4-2B, which is consistent with pAkt expression (Fig. 2A). It has been shown that Akt can control FoxM1 expression in osteosarcoma [Major et al., 2004], and thus we sought to determine whether FoxM1 expression could be controlled by Akt in PCa cells. As expected, over-expression of pAkt by Akt cDNA plasmid increased FoxM1 expression in PC-3 cells (Fig. 2B). However, down-regulation of pAkt by siRNA inhibited FoxM1 expression in PC-3 cells (Fig. 2B). Moreover, we found that LY294002 and Wortmanin, the PI3K inhibitors, eliminated the expression of FoxM1 (Fig. 2C), suggesting that FoxM1 is regulated by Akt pathway in PCa cells, and further suggesting that inactivation of Notch-1 could inactivate Akt which, in turn, leads to the inactivation of FoxM1. To further confirm our results in order to document whether FoxM1 is a downstream target of Akt or not, we examined cell growth, which was reduced in Akt knock-out MEFs (Supplementary Fig. 3A). We also examined the expression of FoxM1 in Akt WT, Akt-1KO, and Akt DKO MEF cell lines, and found that FoxM1 was significantly decreased in Akt DKO cell lines, which was consistent with decreased pAkt pathway (Supplementary Fig. 3B). Further experiments were done in PTEN KO MEF, which showed high expression of pAkt and FoxM1 (Supplementary Fig. 3C), suggesting that Akt regulates the expression of FoxM1.

DOWN-REGULATION OF NOTCH-1 EXPRESSION INHIBITED FoxM1
Next, we assessed whether Notch-1 could regulate the FoxM1 expression because Akt is one of the Notch-1 downstream target genes, which appears to regulate FoxM1. Down-regulation of Notch-1 by siRNA transfection and gamma secretase inhibitor (GSI, L-685,458, DAPT) treatments showed lower expression of Notch-1 protein as confirmed by Western blotting (Fig. 3A,B). We also found
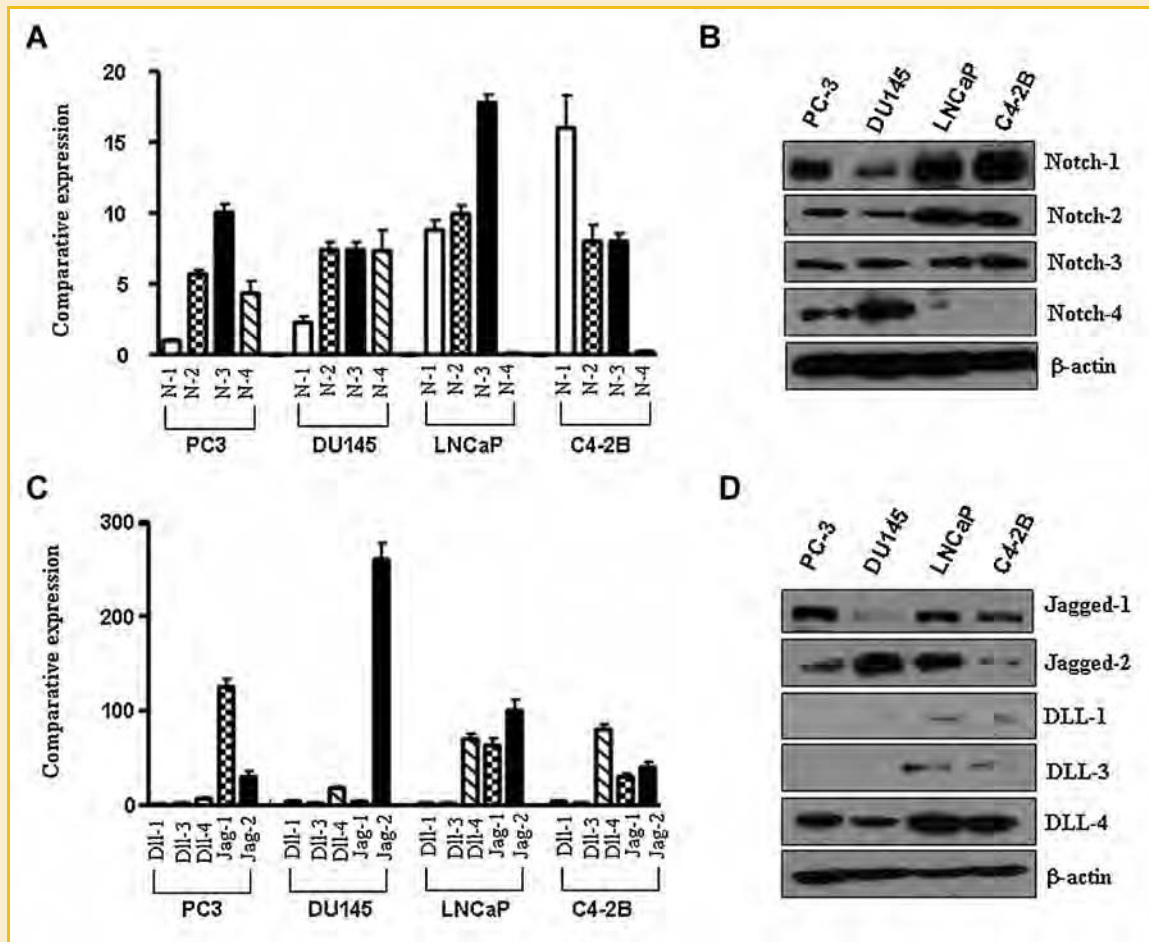
Fig. 1. Notch signaling pathway in PCa cell lines. The baseline expression of Notch signaling pathway was determined in a panel of PCa cell lines using real-time RT–PCR (A,C) and Western blotting analysis (B,D), respectively.

that the inhibition of Notch-1 significantly decreased pAkt and FoxM1 expression in PC-3 and C4-2B cell lines (Fig. 3A,B). Conversely, over-expression of Notch-1 by ICN transfection showed increased expression of FoxM1 at the mRNA and protein levels (Fig. 3B). Moreover, Notch-1 siRNA decreased pAkt and FoxM1 in Akt WT cell lines (Supplementary Fig. 3C). Furthermore, immuno-fluorescent staining showed higher levels of FoxM1 protein in PC-3 ICN cells (Fig. 3C), suggesting that FoxM1 is regulated by Notch-1, which could be due to inactivation of Akt. Although these molecular studies clearly suggest that Notch-1 inactivation could be an important strategy for the prevention of tumor progression and/or therapy, we sought to investigate whether we could find any "natural" non-toxic agent that could down-regulate Notch-1 and consequently could inactivate Akt and FoxM1.

DOWN–REGULATION OF NOTCH–1 EXPRESSION BY GENISTEIN

Our previous studies have shown that genistein inhibited cell growth and induced cell apoptotic death in PCa cells. However, genistein did not inhibit the normal prostate cell growth [Banerjee et al., 2008]. In order to further understand the molecular mechanism involved in genistein-induced apoptosis of PCa cells, alterations in the cell survival pathway were investigated. Notch signaling is over-

expressed in PCa and is involved in the inhibition of apoptosis and potentiation of cell growth and thus considered as a putative target for drug development. Therefore, we investigated whether genistein could regulate Notch signaling pathway. Notch-1 mRNA and protein expression in PCa cell lines treated with genistein for 72 h were assessed. We found that Notch-1 was down-regulated by genistein in all three cell lines (Fig. 4A). To confirm the downstream effect on Notch down-regulation by genistein, we also assessed the expression of Notch-1 target gene pAkt in PCa cells after genistein treatment. We found that genistein inhibited the pAkt expression (Fig. 4A). We also assessed the expression of FoxM1 in PCa cells treated with genistein, and as expected, we found down-regulation of FoxM1 in genistein-treated cells (Fig. 4A). To further confirm our results, we also did immunofluorescent staining. Indeed, we observed lower level of FoxM1 protein in genistein-treated cells (Fig. 4B).

DOWN–REGULATION OF NOTCH–1 EXPRESSION BY siRNA POTENTIATES GENISTEIN–INDUCED CELL GROWTH INHIBITION AND APOPTOSIS

In order to gain further molecular insight, we assessed whether inactivation of Notch-1 by Notch-1-specific siRNA could lead to potentiate the effects of genistein. We found that the down-
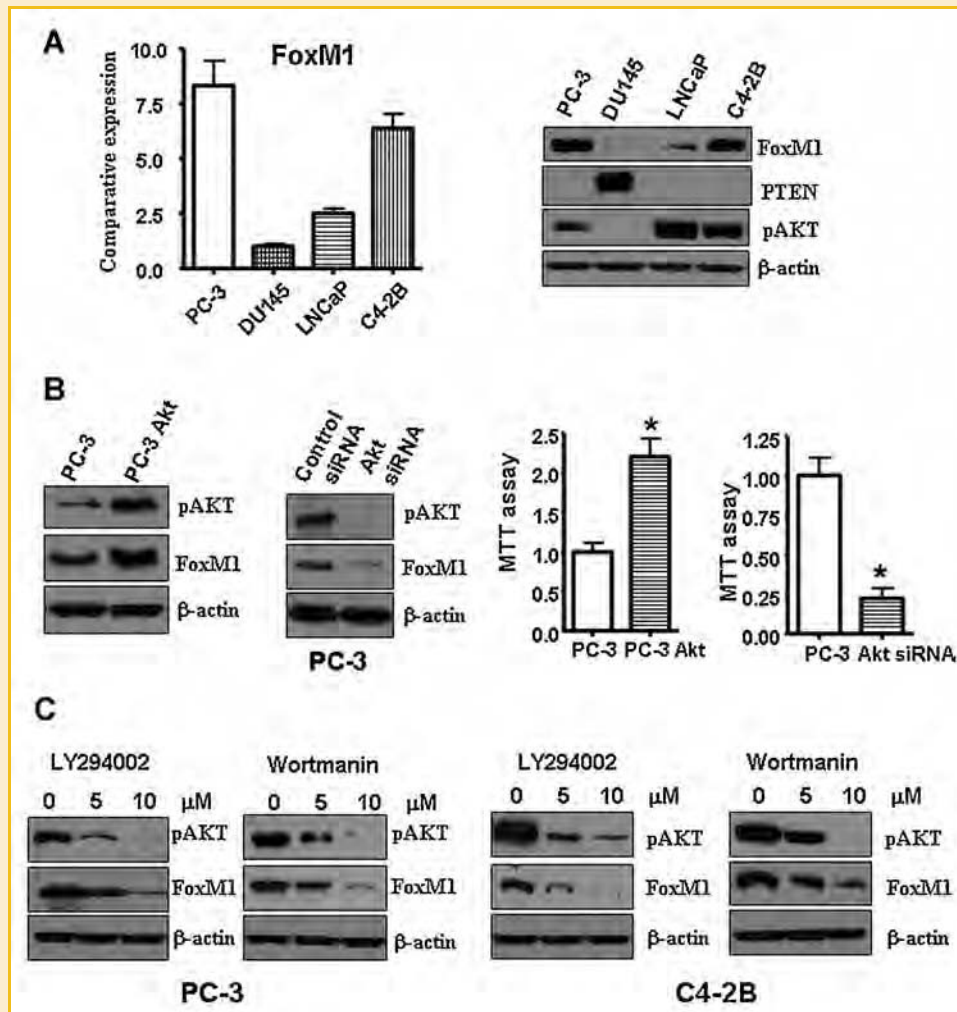
Fig. 2. Down–regulation of Akt inhibited FoxM1. A: The baseline expression of FoxM1 was determined between a panel of PCa cell lines using real–time RT–PCR and Western blotting analysis, respectively. B: Down–regulation of Akt by siRNA inhibited FoxM1 expression, whereas up–regulation of Akt by cDNA plasmid transfection resulted in increased expression of FoxM1. Akt siRNA inhibited cell growth, while Akt cDNA transfection promoted cell growth. C: Inactivation of Akt by PI3K inhibitors (LY294002, Wortmanin) inhibited the expression of pAkt, which was consistent with decreased expression of FoxM1 as assessed by Western blot analysis.

regulation of Notch-1 expression significantly inhibited cell growth induced by genistein (Fig. 5). Genistein plus Notch-1 siRNA inhibited cell growth to a greater degree compared to genistein alone. Moreover, Notch-1 siRNA-transfected PC-3 cells were significantly more sensitive to spontaneous and genistein-induced apoptosis (Fig. 5A). However, over-expression of Notch-1 rescued genistein-induced cell growth inhibition and abrogated genistein-induced apoptosis to a certain degree (Fig. 5B). These results provide molecular evidence suggesting that genistein induced cell growth inhibition and apoptosis is in part mediated through Notch-1 signaling pathway in PCa cells.

GENISTEIN–MEDIATED EFFECTS ON PCa CELLS WERE ENHANCED BY TAXOTERE IN INHIBITING CELL GROWTH AND CAUSING INDUCTION OF APOPTOSIS

First, we found that taxotere inhibited cell growth and induced apoptosis in PC-3, LNCaP, and C4-2B cells (Supplementary Fig. 4). Second, we found that taxotere did not significantly inhibit the

Notch-1 and pAkt expression (data not shown). However, taxotere inhibited FoxM1 expression in all three PCa cells (Fig. 5C). Next, we tested whether genistein could synergize with taxotere leading to enhanced suppression of cell growth as assessed by MTT assay. As can be seen from the results presented in Figure 5D, 30 μM genistein alone or 1 nM taxotere alone caused 40–50% cell growth inhibition, whereas the combination of genistein and taxotere showed drastic inhibition (about 80%) in cell growth, which was also found to be synergistic in inducing apoptotic cell death (Fig. 5D).

GENISTEIN INHIBITED TUMOR GROWTH IN VIVO

To test whether genistein has similar effects in vivo, we conducted an animal experiment using SCID-human model of experimental bone metastasis of PCa. We found that genistein significantly inhibited PC-3 and C4-2B tumor growth, demonstrating an inhibitory effect of genistein in the in vivo model of PCa (Fig. 6A). The body weight of mice in each group did not show any significant difference, suggesting non-toxic nature of genistein.
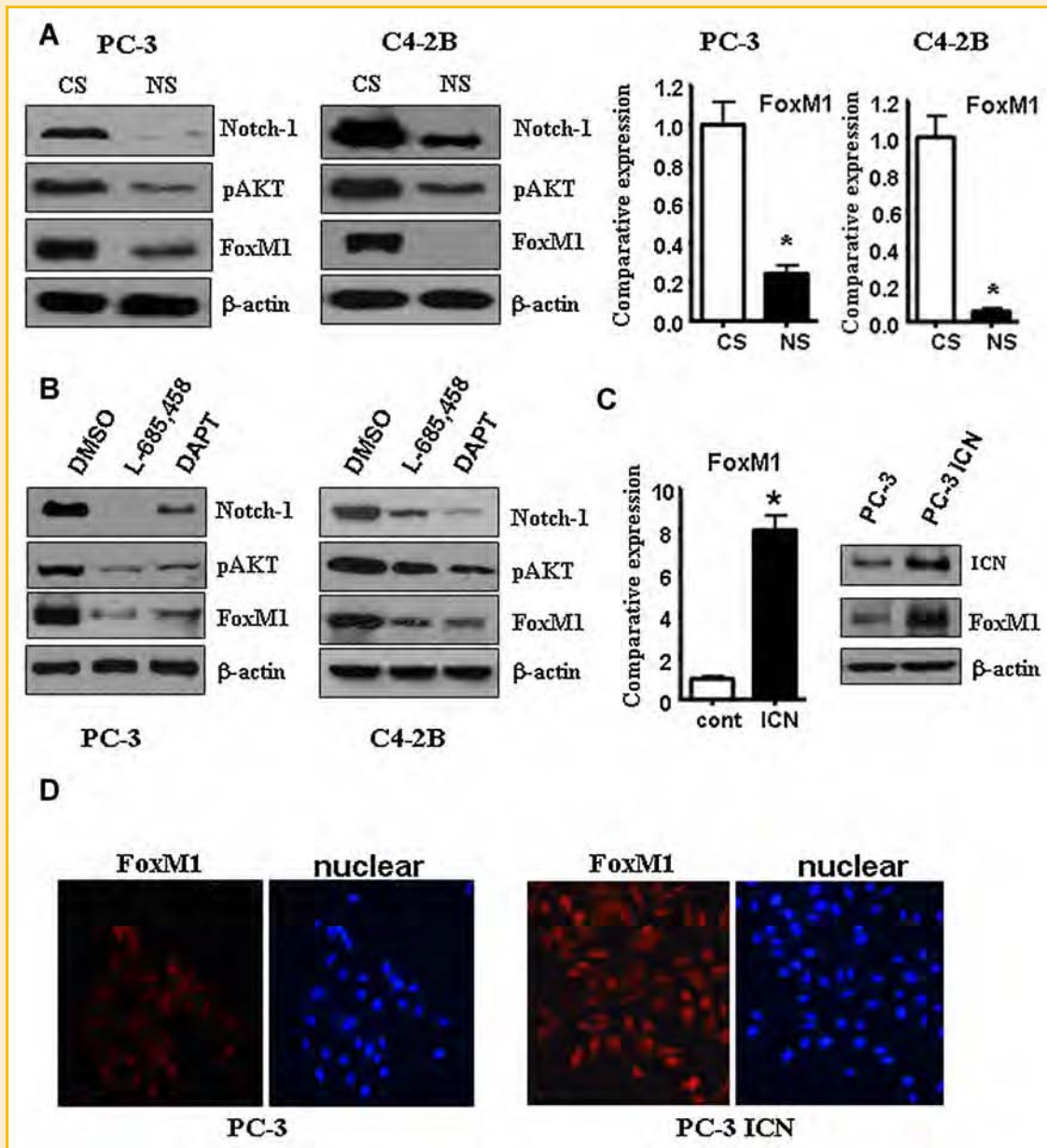
Fig. 3. Inhibition of FoxM1 expression by Notch–1 siRNA and GSI. A: The expression of FoxM1 was detected by Western blotting analysis (left panel) and real–time RT–PCR (right panel) in PCa cells transfected with Notch–1 siRNA. B: The expression of FoxM1 was detected by Western blotting analysis in PCa cells treated with GSI for 72 h. C: The expression of FoxM1 was detected by real–time RT–PCR (left panel) and Western blotting analysis (right panel) in PC–3 ICN cells. D: The PC–3 and PC–3 ICN cells were subjected to immunofluorescent staining using anti–FoxM1 antibody. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In order to further explore the molecular mechanism by which genistein caused anti-tumor activity, we analyzed the gene expression altered by genistein treatment, and we found that genistein inhibited the expression of Notch-1, pAkt, and FoxM1 in tumor remnants (Fig. 6B).

DISCUSSION

Notch signaling plays important roles in maintaining the balance between cell proliferation, differentiation, and apoptosis [Kopan and Ilagan, 2009]. The Notch gene is abnormally activated in many human malignancies [Miele, 2006; Rizzo et al., 2008]. It has been reported that the Notch signaling is involved in PCa cell survival and that Notch signaling pathway components and Notch target genes are up-regulated in PCa [Villaronga et al., 2008; Bin et al., 2009]. Moreover, Notch-1 expression in human PCa tissues increased with increasing tumor grade [Bin et al., 2009]. In our previous studies, we have shown that down-regulation of Notch-1 inhibits cell growth and induced apoptosis in PCa cells [Wang et al., 2010b]. Therefore, the inhibition of Notch signaling is likely to have beneficial effects toward designing strategies for the prevention of tumor progression and/or therapy for PCa.
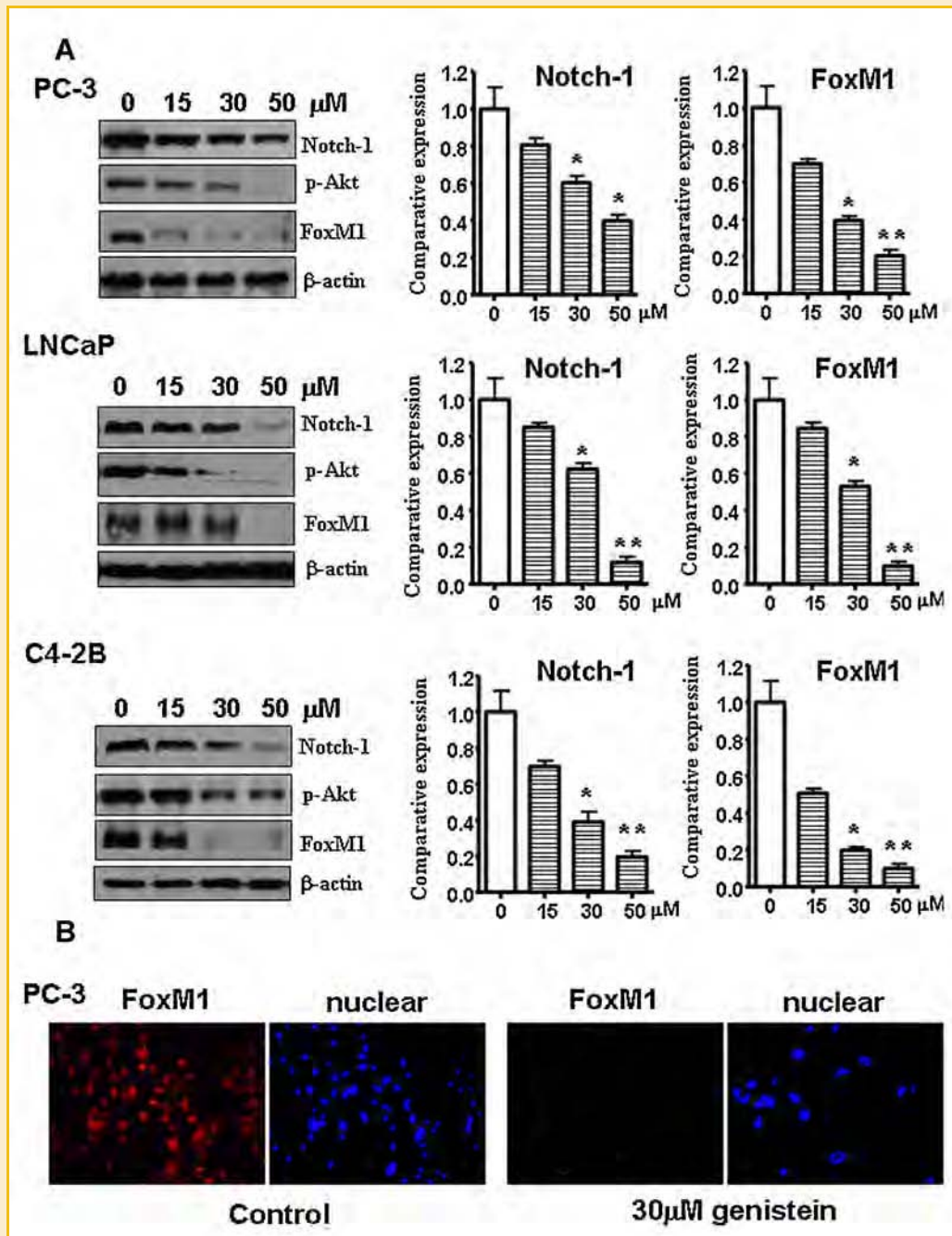
Fig. 4. Inhibition of Notch, Akt, and FoxM1 expression by genistein. A: PCa cells were treated with varied concentrations of genistein for 72 h. Left panel: The expression of Notch, pAkt, and FoxM1 protein was detected by Western blotting analysis. Middle and right panel: Notch-1 mRNA and FoxM1 mRNA were detected by real-time RT-PCR. B: Immunofluorescent staining showing lower levels of FoxM1 protein in the cytoplasm and nucleus in the genistein-treated PC-3 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Although several studies have shown the functional significance of Notch signaling, the role of Notch pathway in prostate carcinogenesis remains poorly understood. Therefore, in the present study, we investigated the mechanisms of Notch-1 in cell proliferation in PCa cells. Recently, Notch has been shown to regulate the Akt pathway. It has been reported that Notch-1 activation enhanced melanoma cell survival via activation of the Akt pathway [Liu et al., 2006]. Palomero et al. [2008] found that

Notch-1 up-regulated the PI3K–Akt pathway, which negatively controls the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-ALL. In the current study, we found that down-regulation of Notch-1 by siRNA or GSI decreased Akt phosphorylation in PCa cells. Recently, Akt pathway has been shown to cross-talk with the FoxM1 pathway [Major et al., 2004; Park et al., 2009] and FoxM1 has been shown to be over-expressed in many human cancers including PCa [Wang et al., 2010a]. Given
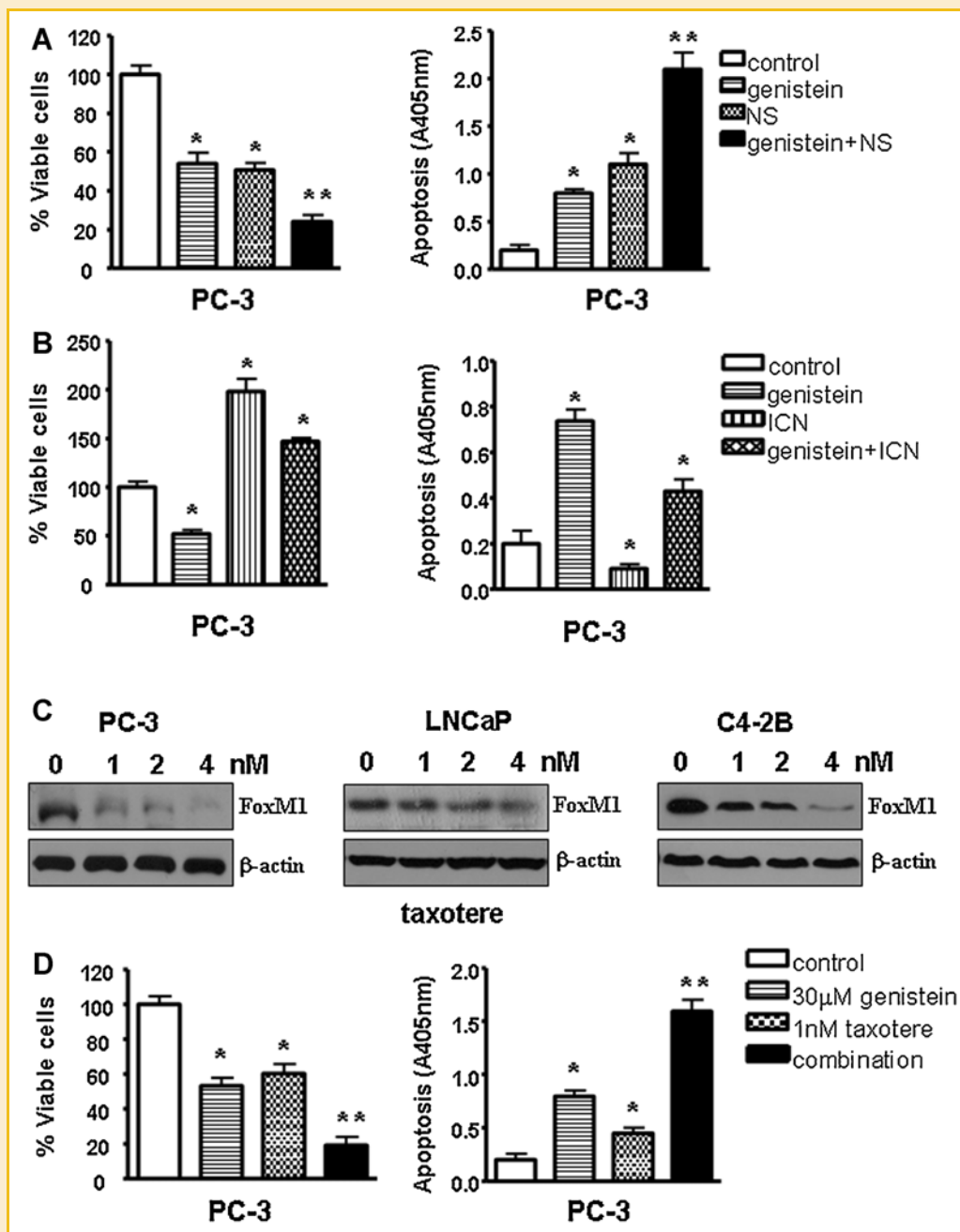
Fig. 5. Down-regulation of Notch-1 by siRNA promotes genistein-induced cell growth inhibition and apoptosis in PC-3 cells. Genistein: 30 μM genistein; NS: Notch-1 siRNA; ICN: ICN cDNA; genistein + siRNA: 30 μM genistein + Notch-1 siRNA; genistein + ICN: 30 μM genistein + ICN cDNA. A: Left panel: Down-regulation of Notch-1 by siRNA significantly inhibited PC-3 cell growth. Genistein plus Notch-1 siRNA inhibited cell growth to a greater degree compared to genistein alone. Right panel: Down-regulation of Notch-1 expression significantly increased apoptosis induced by genistein. Notch-1 siRNA-transfected cells were significantly more sensitive to spontaneous and genistein-induced apoptosis. B: Over-expression of Notch-1 expression significantly promoted cell growth. Over-expression of Notch-1 rescued cells from genistein-induced cell growth inhibition. Over-expression of Notch-1 by Notch-1 cDNA transfection abrogated genistein-induced apoptosis to a certain degree. C: The expression of FoxM1 was detected by Western blotting analysis in PCa cells treated with different concentrations of taxotere for 72 h. D: Left panel: Genistein synergize with taxotere leading to enhanced suppression of cell growth as assessed by MTT assay. Right panel: Genistein combined with taxotere led to synergistic induction of apoptotic cell death.

the emerging data describing the important role of FoxM1 in the progression of human cancers, Radhakrishnan et al. [2006] have rightly pointed out that it should be possible to target multiple facets of tumorigenesis by inhibiting only this single transcription factor. In our study, we investigated whether Akt could regulate FoxM1 expression in PCa cells. Indeed, we found that down-regulation of Akt by siRNA inhibited FoxM1 expression, whereas over-expression of Akt increased FoxM1 expression. Moreover, inactivation of Akt by LY294002 and Wortmanin decreased FoxM1 expression. Furthermore, we found that down-regulation of Notch-1 by siRNA
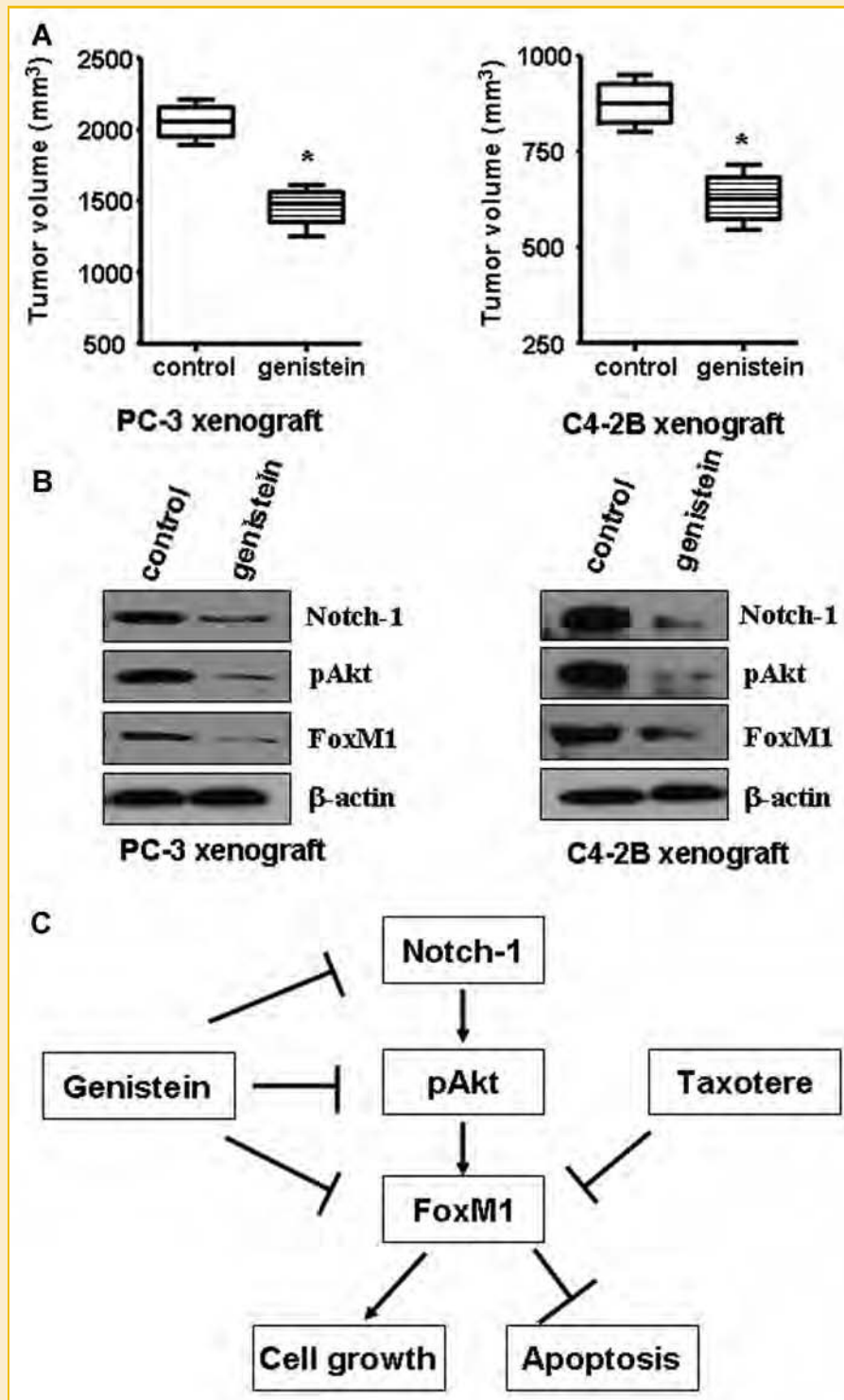
Fig. 6. Genistein inhibited tumor growth in animal model. A: Inhibitory effects of genistein on the growth of tumors formed by PC–3 or C4–2B cells in SCID–human mice (control, n = 7; genistein, n = 7). Comparison of the tumor volumes in each group on the day when all mice were sacrificed (*P < 0.05, genistein vs. control). B: Genistein inhibited the expression of Notch–1, pAkt, and FoxM1 in tumor remnants as assessed by Western blot analysis. C: The schematic presentation of our proposed mechanism of how genistein inhibits cell growth and induces apoptosis.

or GSI decreased FoxM1 expression. We also found that FoxM1 was decreased significantly in Akt DKO cell lines, which was consistent with decreased pAkt pathway. These results suggest that Notch-1 could mediate FoxM1 signaling through Akt.

In the present study, we also found that a ''natural agent'' discovered from soybean such as genistein could inhibit Notch-1 activity in PCa cells, suggesting that this could be one mechanism by which genistein inhibits cell growth and induces apoptosis. Moreover, down-regulation of Notch-1 by siRNA together with genistein treatment inhibited cell growth and induced apoptosis to a greater degree in PC-3 cells compared to genistein treatment alone, which suggest that inactivation of Notch-1 could be mechanistically linked with the biological effects of genistein. Moreover, we have shown, for the first time, that genistein could inhibit the expression of FoxM1 in vitro and in vivo in PCa. The similar results were also found in pancreatic cancer cells and its orthotopic animal model in vivo (data not shown), and the anti-tumor activity of genistein was correlated with decreased expression of Notch-1, pAkt, and FoxM1 in tumor remnants (data not shown), which was consistent with in vivo findings in PCa. Therefore, genistein-mediated cell growth inhibition could be partly mediated via inactivation of FoxM1 activity. In view of these findings, we strongly believe that inactivation of FoxM1 by genistein appears to be mechanistically linked with genistein-induced cell growth inhibition and apoptosis in PCa cells. It has been found that taxotere down-regulated some genes for cell proliferation, transcription factors, and oncogenesis, and up-regulated some genes related to induction of apoptosis and cell-cycle arrest in PCa cells. Here, we found that taxotere alone could down-regulate FoxM1 and the combination of taxotere with genistein showed greater down-regulation in the expression of FoxM1, which appears to be consistent with the inhibition of cell growth and induction of apoptosis in PCa, further suggesting that the combination of taxotere with genistein could be an useful therapeutic strategy for the treatment of metastatic PCa.

In summary, we presented experimental evidence which strongly suggest that the role of Notch-1 down-regulation could be a potential anti-tumor and anti-metastatic approach toward the treatment of PCa. Moreover, our current data provided mechanistic information showing that genistein exerts its pro-apoptotic effects on PCa cells, which is in part due to inactivation of Notch-1, Akt, and FoxM1 signaling (Fig. 6C). On the basis of our results, we propose a hypothetical pathway by which genistein inhibits cell growth of PCa cells although further in-depth studies are needed to ascertain how genistein regulates these pathways. However, we believe that Notch-1, Akt, and FoxM1 are intimate partners of the same crime of tumor aggressiveness, and thus targeted inactivation of these pathways by genistein together with taxotere may prove to be a novel therapeutic approach for the treatment of PCa in the future.

ACKNOWLEDGMENTS

REFERENCES

Banerjee S, Zhang Y, Ali S, Bhuiyan M, Wang Z, Chiao PJ, Philip PA, Abbruzzese J, Sarkar FH. 2005. Molecular evidence for increased antitumor activity of gemcitabine by genistein in vitro and in vivo using an orthotopic model of pancreatic cancer. Cancer Res 65:9064–9072.

Banerjee S, Hussain M, Wang Z, Saliganan A, Che M, Bonfil D, Cher M, Sarkar FH. 2007a. In vitro and in vivo molecular evidence for better therapeutic efficacy of ABT-627 and taxotere combination in prostate cancer. Cancer Res 67:3818–3826.

Banerjee S, Zhang Y, Wang Z, Che M, Chiao PJ, Abbruzzese JL, Sarkar FH. 2007b. In vitro and in vivo molecular evidence of genistein action in augmenting the efficacy of cisplatin in pancreatic cancer. Int J Cancer 120:906–917.

Banerjee S, Li Y, Wang Z, Sarkar FH. 2008. Multi-targeted therapy of cancer by genistein. Cancer Lett 269:226–242.

Bektas N, Haaf A, Veeck J, Wild PJ, Luscher-Firzlaff J, Hartmann A, Knuchel R, Dahl E. 2008. Tight correlation between expression of the Forkhead transcription factor FOXM1 and HER2 in human breast cancer. BMC Cancer 8:1–9.

Bin HB, Adhami VM, Asim M, Siddiqui IA, Bhat KM, Zhong W, Saleem M, Din M, Setaluri V, Mukhtar H. 2009. Targeted knockdown of Notch1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. Clin Cancer Res 15:452–459.

Chandran UR, Ma C, Dhir R, Bisceglia M, Lyons-Weiler M, Liang W, Michalopoulos G, Becich M, Monzon FA. 2007. Gene expression profiles of prostate cancer reveal involvement of multiple molecular pathways in the metastatic process. BMC Cancer 7:1–21.

Chiuri VE, Silvestris N, Lorusso V, Tinelli A. 2009. Efficacy and safety of the combination of docetaxel (taxotere) with targeted therapies in the treatment of solid malignancies. Curr Drug Targets 10:982–1000.

Falci C, Morello E, Droz JP. 2009. Treatment of prostate cancer in unfit senior adult patients. Cancer Treat Rev 35:522–527.

Gartel AL. 2008. FoxM1 inhibitors as potential anticancer drugs. Expert Opin Ther Targets 12:663–665.

Gartel AL. 2010. A new target for proteasome inhibitors: FoxM1. Expert Opin Invest Drugs 19:235–242.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. 2009. Cancer statistics, 2009. CA Cancer J Clin 59:225–249.

Kalin TV, Wang IC, Ackerson TJ, Major ML, Detrisac CJ, Kalinichenko VV, Lyubimov A, Costa RH. 2006. Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. Cancer Res 66:1712–1720.

Kopan R, Ilagan MX. 2009. The canonical Notch signaling pathway: Unfolding the activation mechanism. Cell 137:216–233.

Leong KG, Gao WQ. 2008. The Notch pathway in prostate development and cancer. Differentiation 76:699–716.

Li Y, Ahmed F, Ali S, Philip PA, Kucuk O, Sarkar FH. 2005a. Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. Cancer Res 65:6934–6942.

Li Y, Hong X, Hussain M, Sarkar SH, Li R, Sarkar FH. 2005b. Gene expression profiling revealed novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells. Mol Cancer Ther 4:389–398.

Li Y, Hussain M, Sarkar SH, Eliason J, Li R, Sarkar FH. 2005c. Gene expression profiling revealed novel mechanism of action of taxotere and furtulon in prostate cancer cells. BMC Cancer 5:1–13.

Li Y, Kucuk O, Hussain M, Abrams J, Cher ML, Sarkar FH. 2006. Antitumor and antimetastatic activities of docetaxel are enhanced by genistein through regulation of osteoprotegerin/receptor activator of nuclear factor-kappaB

(RANK)/RANK ligand/MMP-9 signaling in prostate cancer. Cancer Res 66:4816–4825.

Li Q, Zhang N, Jia Z, Le X, Dai B, Wei D, Huang S, Tan D, Xie K. 2009. Critical role and regulation of transcription factor FoxM1 in human gastric cancer angiogenesis and progression. Cancer Res 69:3501–3509.

Liu ZJ, Xiao M, Balint K, Smalley KS, Brafford P, Qiu R, Pinnix CC, Li X, Herlyn M. 2006. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. Cancer Res 66:4182–4190.

Major ML, Lepe R, Costa RH. 2004. Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators. Mol Cell Biol 24:2649–2661.

Miele L. 2006. Notch signaling. Clin Cancer Res 12:1074–1079.

Miele L, Miao H, Nickoloff BJ. 2006. Notch signaling as a novel cancer therapeutic target. Curr Cancer Drug Targets 6:313–323.

Osipo C, Golde TE, Osborne BA, Miele LA. 2008. Off the beaten pathway: The complex cross talk between Notch and NF-kappaB. Lab Invest 88:11–17.

Palomero T, Dominguez M, Ferrando AA. 2008. The role of the PTEN/AKT pathway in NOTCH1-induced leukemia. Cell Cycle 7:965–970.

Pandit B, Gartel AL. 2010. New potential anti-cancer agents synergize with bortezomib and ABT-737 against prostate cancer. Prostate 70:825–833.

Park HJ, Carr JR, Wang Z, Nogueira V, Hay N, Tyner AL, Lau LF, Costa RH, Raychaudhuri P. 2009. FoxM1, a critical regulator of oxidative stress during oncogenesis. EMBO J 28:2908–2918.

Radhakrishnan SK, Bhat UG, Hughes DE, Wang IC, Costa RH, Gartel AL. 2006. Identification of a chemical inhibitor of the oncogenic transcription factor forkhead box m1. Cancer Res 66:9731–9735.

Rizzo P, Osipo C, Foreman K, Golde T, Osborne B, Miele L. 2008. Rational targeting of Notch signaling in cancer. Oncogene 27:5124–5131.

Shou J, Ross S, Koeppen H, de Sauvage FJ, Gao WQ. 2001. Dynamics of notch expression during murine prostate development and tumorigenesis. Cancer Res 61:7291–7297.

Villaronga MA, Bevan CL, Belandia B. 2008. Notch signaling: A potential therapeutic target in prostate cancer. Curr Cancer Drug Targets 8:566–580.

Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y, Sarkar FH. 2006a. Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{kappa}B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 66:2778–2784.

Wang Z, Zhang Y, Li Y, Banerjee S, Liao J, Sarkar FH. 2006b. Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. Mol Cancer Ther 5:483–493.

Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH. 2007. Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer Res 67: 8293–8300.

Wang Z, Li Y, Banerjee S, Sarkar FH. 2008. Exploitation of the Notch signaling pathway as a novel target for cancer therapy. Anticancer Res 28:3621–3630.

Wang Z, Azmi AS, Ahmad A, Banerjee S, Wang S, Sarkar FH, Mohammad RM. 2009. TW-37, a small-molecule inhibitor of Bcl-2, inhibits cell growth and induces apoptosis in pancreatic cancer: Involvement of Notch-1 signaling pathway. Cancer Res 69:2757–2765.

Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH. 2010a. Forkhead box M1 transcription factor: A novel target for cancer therapy. Cancer Treat Rev 36:151–156.

Wang Z, Li Y, Banerjee S, Kong D, Ahmad A, Nogueira V, Hay N, Sarkar FH. 2010b. Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-kappaB signaling pathways. J Cell Biochem 109:726–736.

Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A, Osborne BA, Gottipati S, Aster JC, Hahn WC, Rudolf M, Siziopikou K, Kast WM, Miele L. 2002. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 8:979–986.

Zhang Y, Wang Z, Ahmed F, Banerjee S, Li Y, Sarkar FH. 2006. Down-regulation of Jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells. Int J Cancer 119:2071–2077.

Down-Regulation of Notch-1 and Jagged-1 Inhibits Prostate Cancer Cell Growth, Migration and Invasion, and Induces Apoptosis Via Inactivation of Akt, mTOR, and NF-κB Signaling Pathways

Zhiwei Wang,[1]* Yiwei Li,[1] Sanjeev Banerjee,[1] Dejuan Kong,[1] Aamir Ahmad,[1] Veronique Nogueira,[2] Nissim Hay,[2] and Fazlul H Sarkar[1]**

[1]*Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201*
[2]*Department of Biochemistry and Molecular Genetics, University of Illinois, Chicago, Illinois 60612*

ABSTRACT

Notch signaling is involved in a variety of cellular processes, such as cell fate specification, differentiation, proliferation, and survival. Notch-1 over-expression has been reported in prostate cancer metastases. Likewise, Notch ligand Jagged-1 was found to be over-expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues, suggesting the biological significance of Notch signaling in prostate cancer progression. However, the mechanistic role of Notch signaling and the consequence of its down-regulation in prostate cancer have not been fully elucidated. Using multiple cellular and molecular approaches such as MTT assay, apoptosis assay, gene transfection, real-time RT-PCR, Western blotting, migration, invasion assay and ELISA, we found that down-regulation of Notch-1 or Jagged-1 was mechanistically associated with inhibition of cell growth, migration, invasion and induction of apoptosis in prostate cancer cells, which was mediated via inactivation of Akt, mTOR, and NF-κB signaling. Consistent with these results, we found that the down-regulation of Notch-1 or Jagged-1 led to decreased expression and the activity of NF-κB downstream genes such as MMP-9, VEGF, and uPA, contributing to the inhibition of cell migration and invasion. Taken together, we conclude that the down-regulation of Notch-1 or Jagged-1 mediated inhibition of cell growth, migration and invasion, and the induction of apoptosis was in part due to inactivation of Akt, mTOR, and NF-κB signaling pathways. Our results further suggest that inactivation of Notch signaling pathways by innovative strategies could be a potential targeted approach for the treatment of metastatic prostate cancer. J. Cell. Biochem. 109: 726–736, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: NOTCH-1; JAGGED-1; PROSTATE CANCER; CELL GROWTH; INVASION

Prostate cancer has become a significant health problem because it is one of the most frequently diagnosed tumors in men and the second leading cause of cancer-related death in the United States (Jemal et al., 2009). Despite an initial efficacy of androgen-deprivation therapy, most patients with prostate cancer progress from androgen-dependent status to hormone-refractory prostate cancer also known as castrate resistant cancer for which there is no curative therapy. Therefore, development of novel strategies for the treatment of prostate cancer is highly desirable for improving the survival outcome of this deadly disease.

Among many molecular targets, Notch signaling is very attractive because it is involved in a variety of cellular processes, such as cell fate specification, differentiation, proliferation, and survival. Four Notch receptors (Notch 1–Notch 4) and five ligands (Jagged-1, Jagged-2, Delta-1, Delta-3, and Delta-4) have been described in mammals (Wang et al., 2008). Binding of ligand to its receptor induces metalloproteinase-mediated and gamma secretase-mediated cleavage of the Notch receptor. The Notch intracellular domain (ICN) is released from the plasma membrane and translocates into the nucleus, where it forms a complex with the

members of the CSL transcription factor family. This complex mediates the transcription of target genes such as Hes-1 (hairy enhancer of split), cyclin D, Hey-1, and others (Miele, 2006; Miele et al., 2006). Because Notch signaling plays important roles in the cellular developmental pathway including proliferation and apoptosis, activation of Notch signaling pathways are associated with tumorigenesis. It has been reported that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands were found in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and Large-cell lymphomas and pancreatic cancer (Miele and Osborne, 1999; Miele, 2006; Miele et al., 2006; Rizzo et al., 2008; Villaronga et al., 2008; Kopan and Ilagan, 2009).

Moreover, emerging evidence suggest that Notch signaling pathways also play important role in prostate development and progression, especially because Notch signaling pathway was found to be over-expressed in prostate cancer cell lines (Shou et al., 2001; Zhang et al., 2006; Leong and Gao, 2008; Bin et al., 2009). In addition, Notch-1 over-expression has been reported in prostate cancer metastases. Specifically, bone metastases from prostate cancer patients expressed Notch-1 protein in osteoblastic prostate cancer metastatic cells (Zayzafoon et al., 2004). Likewise Notch ligand Jagged-1 was found to be highly expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostate tissues, and high Jagged-1 expression in a subset of clinically localized tumors was found to be significantly associated with tumor recurrence (Santagata et al., 2004). Recently, Hafeez et al. reported that silencing of Notch-1 inhibited invasion of prostate cancer cells by inhibiting the expression of MMP-9 and uPA (Bin et al., 2009). However, it is not clear whether Jagged-1 plays a role in prostate cancer progression. Moreover, the precise molecular mechanism by which activation of Notch signaling pathway leads to prostate cancer cell growth and invasion, and the mechanistic consequence of the down-regulation of Notch signaling in prostate cancer has not been fully understood.

In the present study, we sought to gain molecular evidence in support of the mechanistic consequence of Notch-1 and Jagged-1 down-regulation in cell growth, migration, invasion and apoptosis using human prostate cancer cells. Our results show that down-regulation of Notch-1 and Jagged-1 could be an effective approach for inhibiting cell growth, migration and invasion, and inducing apoptotic cell death, which was associated with inactivation of Akt, mTOR (mammalian Target of Rapamycin) and NF-κB, and the expression and activity of NF-κB target genes such as, MMP-9, uPA. Our results suggest that inactivation of Notch signaling pathways by innovative strategies could be a potential targeted approach for the treatment of metastatic prostate cancer.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL REAGENTS

Human PC cell lines, including PC-3, DU145, LNCaP, and C4-2B cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, and 2.5 mM glutamine in a humidified incubator with 5% CO_2 and 95% air at 37°C. Primary mouse embryo fibroblasts (MEFs) were harvested from E13.5 embryos as described previously (Skeen et al., 2006). *Akt* WT, *Akt1*$^{-/-}$ (Akt1 KO), *Akt1/2*$^{-/-}$ (Akt DKO) MEF were cultured in DMEM with 10% FBS. Primary antibodies for full-length Notch-1 (H-130), cleaved Notch-1 (C-20), Jagged-1, uPA, uPAR, VEGF, and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against mTOR, phospho-mTOR (Ser^{2448}), p70S6K, phospho-p70S6K (Thr^{389}), 4E-BP1 (eukaryotic translation initiation factor 4E–binding protein 1), phospho-4E-BP1 (Thr^{37}/Thr^{46}), Akt, and phospho-Akt (Ser^{473}) were purchased from Cell Signaling Technology. All secondary antibodies were obtained from Pierce (Rockford, IL). The monoclonal antibody to β-actin, Gamma secretase inhibitor and PI3K inhibitor, LY294002, were purchased from Sigma–Aldrich. Gamma secretase inhibitor L-685,458 and DAPT were obtained from Calbiochem (San Diego, CA). Lipofecta-mine 2000 was purchased from Invitrogen. Chemiluminescence detection of proteins was done with the use of a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO).

PLASMIDS AND TRANSFECTION PROCEDURES

Notch-1, Jagged-1 and control siRNA were obtained from Santa Cruz Biotechnology. PC-3 cells were transfected with Notch-1 siRNA, Jagged-1 siRNA and siRNA control, respectively, using Lipofectamine 2000 as described earlier (Wang et al., 2006a). The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain (NICD) was a kind gift of L. Miele {Department of Medicine and Pharmacology, University of Mississippi Cancer Institute, Jackson, MS). PC-3 cells were transfected with human Notch-1 ICN, Akt, p65 or vector alone (pcDNA3), respectively.

LUCIFERASE REPORTER ASSAY

The Notch-1 siRNA and Jagged-1 siRNA transfected PC-3 cells were transiently transfected with CBF-1 luciferase constructs. The Notch-CBF-1 reporter, 4× wild-type CBF-1 Luc, which contains four tandem repeats of the consensus CBF-1 DNA binding sequence, GTGGGAA and N-terminally tagged FLAG CBF-1, was generous gifts from Dr. Diane Hayward (Johns Hopkins University School of Medicine, USA). Wild-type (4× WT CBF1 luc) CBF-1 luciferase reporter plasmid was co-transfected with β-galactosidase using Lipofectamine (Invitrogen). CMV-β-gal reporter construct transfec-tion was used for normalization of transfection efficiency. Luciferase and β-galactosidase assay (Promega) were done in accordance with the manufacturer's instructions. Luciferase activity was expressed relative to β-galactosidase activity.

CELL GROWTH INHIBITION STUDIES BY 3–(4,5-DIMETHYLTHIAZOL-2–YL)–2,5–DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

The transfected prostate cancer cells (5×10^3) were seeded in a 96-well culture plate and subsequently incubated with MTT reagent (0.5 mg/ml) at 37°C for 2 h and MTT assay was performed as described earlier (Wang et al., 2006b). In addition to the above assay, we have also done clonogenic assay for assessing the effects of treatment as shown below.

CLONOGENIC ASSAY

To test the survival of transfected cells, siRNA transfected PC-3 cells were trypsinized, and the viable cells were counted (trypan blue exclusion) and plated in 100 mm Petri dishes in a range of 100–1,000 cells to determine the plating efficiency as well as for assessing the effects of transfection on clonogenic survival. The cells were then incubated for 10–12 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator. The colonies were stained with 2% crystal violet and counted. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS

The Cell Death Detection ELISA Kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, transfected cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis as described earlier (Wang et al., 2006b).

REAL–TIME REVERSE TRANSCRIPTION–PCR ANALYSIS FOR GENE EXPRESSION STUDIES

The total RNA from transfected cells was isolated by Trizol (Invitrogen) according to the manufacturer's protocols. One microgram of total RNA from each sample was subjected to first strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA). RT reaction was performed at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. The primers used in the PCR reaction for Notch-1, Jagged-1, MMP-9, VEGF, uPA, and β-actin were described before (Wang et al., 2006a, 2007). Real-time PCR amplications were performed as described earlier (Wang et al., 2006b).

PREPARATION OF NUCLEAR LYSATES

Cells were lysed in lysis buffer (0.08 M KCl, 35 mM HEPES, pH 7.4, 5 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 25 mM $CaCl_2$, 0.15 M sucrose, 2 mM phenylmethylsulfonyl fluoride, 8 mM dithiothreitol) and frozen at −80°C overnight. The cell suspension was thawed and passed through a 28-gauge needle three times. A small aliquot of the cells were checked for cell membrane breakage using trypan blue. Then the cell suspension was centrifuged, and the pellet was suspended in lysis buffer, and the nuclei were lysed by sonication. After centrifugation, supernatant was saved as nuclear lysate.

WESTERN BLOT ANALYSIS

Whole cell lysate of the cells was prepared by sonicating the cells lysed in 62 mM Tris–HCl and 2% SDS. In another set of experiments, cytoplasmic and nuclear proteins were also extracted. The protein concentration was measured by the BCA protein assay (Pierce). Total proteins were fractionated using SDS–PAGE and transferred onto a nitrocellulose membrane for Western blotting as described before (Wang et al., 2006a).

IMMUNOFLUORESCENCE STAINING

The transfected cells were plated on cover slips in each well of an 8-well chamber for 48 h. Cells were then fixed with paraformaldehyde for 15 min, rinsed with PBS, and incubated with 5% goat serum for 30 min. The cells were then incubated with anti-Notch-1, anti-Jagged-1, and anti-pAkt antibody for 2 h, respectively. After washing with PBS, the cells were incubated with FITC-conjugated secondary antibody for 45 min and washed with PBS. Cell images were observed under a fluorescent microscope.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts were prepared from treated samples and electrophoretic mobility shift assay was done by incubating 10 µg nuclear extract with IRDye-700-labeled NF-κB oligonucleotide as described earlier (Wang et al., 2006a). The DNA-protein complex formed was visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

UROKINASE PLASMINOGEN ACTIVATOR (uPA) ACTIVITY ASSAY

The culture medium of the transfected cells grown in 6-well plates was collected. After collection, the medium was spun at 800g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for uPA assay later or assayed immediately using commercially available ELISA kits (American Diagnostica, Inc., Stamford, CT).

MMP-9 ACTIVITY ASSAY

The transfected cells were seeded in 6 well plates and incubated at 37°C. After 24 h, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 48 h. MMP-9 activity in the medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay Kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol.

VEGF ASSAY

The transfected cells were seeded in 6-well plates (1.0×10^5 cells per well) and incubated at 37°C. After 24 h, the cell culture supernatant was harvested and cell count was performed after trypsinization. After collection, the medium was spun at 800g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for later VEGF assay or assayed immediately using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN).

CELL MIGRATION AND INVASION ASSAY

Cell migration was assessed using 24-well inserts (BD Biosciences, Bedford, MA) with 8 µm pores according to the manufacturer's protocol. The invasive activity of the siRNA transfected cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences) as described before. Briefly, transfected PC-3 cells (5×10^4) with serum free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 24 h of incubation, the cells in the upper chamber were removed, and the cells, which invaded through matrigel matrix membrane, were stained with 4 µg/ml Calcein AM in Hanks buffered saline at 37°C for 1 h. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

DENSITOMETRIC AND STATISTICAL ANALYSIS

The statistical significance of differential findings between experimental groups and control was determined by Student's t-test. P values <0.05 were considered statistically significant.

RESULTS

NOTCH SIGNALING PATHWAY IN PROSTATE CANCER CELLS

The baseline expression of the Notch signaling was determined in a panel of human prostate cancer cell lines that included PC-3, DU145, LNCaP, and C4-2B. The assays also included the expression of Notch activated downstream signaling proteins (phosphorylated Akt and mTOR). The results showed that the Notch signaling pathway was frequently but differentially dysregulated among different human prostate cancer cell lines. Specifically, the expression of Jagged-1 was highest in PC-3 cell line (Supplemental Fig. 1A,B). The higher expression of Notch-1 (full size and cleaved size) was found in LNCaP and C4-2B cell lines (Supplemental Fig. 1A,B). It is important to note that we focused our studies on the cleaved Notch-1 because it is the active functional form of Notch. Therefore, Notch-1 in our all figure legends means active cleaved Notch-1, except Supplementary Figure 1B, which has been labeled appropriately. We also found that high expression of pAkt and its downstream signaling mTOR pathway in LNCaP and C4-2B cells, suggesting that Notch signaling seems to regulate the Akt and mTOR pathways.

In the present study, we choose the PC-3 cell line to detect whether Notch can regulate Akt and mTOR pathway especially because PC-3 cells showed higher expression of Jagged-1 and moderate levels of Notch-1. In addition, the efficacy of Notch-1 siRNA for knockdown of Notch-1 was better in PC-3 cells compared to LNCaP and C4-2B cells, which was in part due to higher levels of Notch-1 expression and the siRNA approach was not adequate. Moreover, LNCaP and C4-2B are androgen receptor (AR) expressing cell lines and since androgen can regulate the Notch expression especially Notch-2 and Jagged-1 were found to be up-regulated in LNCaP cells after androgen exposure (Martin et al., 2004) and we also found that the expression of Notch-1 and its target genes (Hey-1 and Bcl-2) was up-regulated in PC-3 AR stable transfected cells (Supplemental Fig. 1D). Furthermore, in DU-145 the expression of pAkt and mTOR signaling was barely detectable and, thus, this cell line was not useful for our purposes for investigation of the cross-talk between Notch and Akt. All these results prompted use to use PC-3 cells for the current study; however, further in-depth studies would be needed to ascertain the precise molecular regulation of Notch and AR and their cross-talks in the future for elucidating the role of Notch in cell growth, invasion and angiogenesis of AR expression cell lines, which is being planned for our future studies.

DOWN-REGULATION OF NOTCH-1 AND JAGGED-1 EXPRESSION BY siRNA INHIBITED CELL GROWTH AND INDUCED APOPTOSIS

We have previously reported that Notch-1 and Jagged-1 are highly expressed in PC-3 prostate cancer cell line (Zhang et al., 2006). To determine whether Notch signaling pathway could be an effective therapeutic target for prostate cancer, the biological effect of down-

regulation of Notch signaling pathway using Notch-1 and Jagged-1 siRNAs was examined by assessing cell growth of the prostate cancer cells. The effect of siRNA for knocking down Notch-1 and Jagged-1 at mRNA and protein levels was confirmed by real-time RT-PCR and Western blotting. We found that both Notch-1 and Jagged-1 at mRNA level and protein levels were barely detectable in Notch-1 siRNA and Jagged-1 siRNA transfected cells, respectively, compared to siRNA control transfected cells (Fig. 1A). We also found that the expression of Notch-1 downstream gene Hes-1 and Hey-1 was down-regulated by Notch-1 siRNA or Jagged-1 siRNA (Fig. 1A). Although Notch-1 siRNA and Jagged-1 siRNA transfection resulted in a decrease in the cleaved form of Notch-1 by Western blotting, we
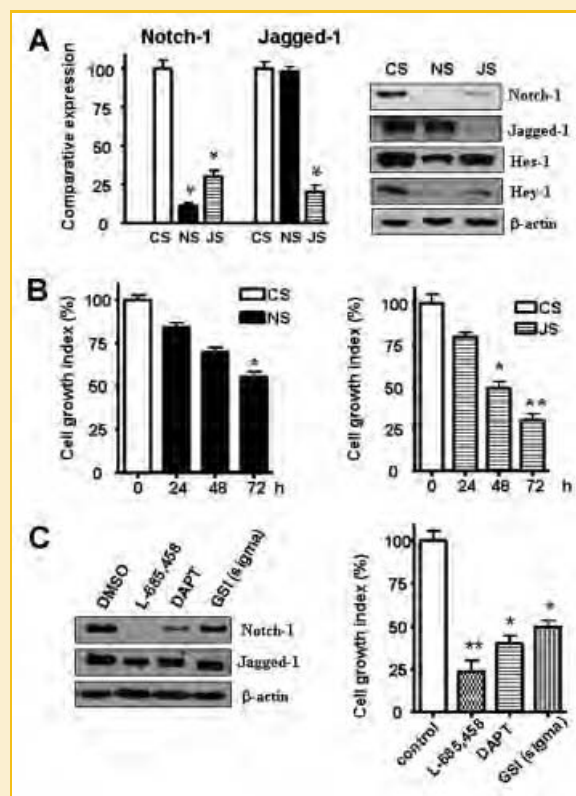

Fig. 1.   Effects of down-regulation of Notch-1 expression on PC-3 prostate cancer cell growth. CS: Control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. $^*P < 0.05$, $^{**}P < 0.01$ relative to control. A: Left, Real-time RT-PCR was done to detect the Notch-1 and Jagged-1 mRNA level. We found that Notch-1 and Jagged-1 mRNA levels were down-regulated by Notch-1 and Jagged-1 siRNA, respectively. The results are presented as the mean of three independent experiments with error bars representing standard deviation (SD). Right, Notch-1, Jagged-1, Hes-1 and Hey-1 protein levels in Notch-1 and Jagged-1 siRNA transfected PC-3 cells were determined using Western Blot analysis. We found that these proteins were down-regulated upon Nocth-1 and Jagged-1 siRNA transfection, respectively. B: Inhibition of cancer cell growth by Notch-1 siRNA and Jagged-1 siRNA tested by MTT assay. The results were plotted as means ± SD of three separate experiments having six determinations per experiment for each experimental condition. C: L-685,458 and DAPT were γ-secretase inhibitors, which prevent the cleavage of the Notch receptor, blocking Notch signal transduction. Left, Western blotting assay showing that GSI inhibited the Notch-1 expression. Right, Inhibition of cancer cell growth by GSI for 72 h as assessed by MTT assay.

wanted to determine if this protein was functional. Thus, we carried out a standard CBF-1 binding luciferase reporter assay. NICD binds with CBF-1 and other proteins to form a DNA binding complex. This complex activates the transcription of target genes. As expected, we found that Notch-1 siRNA and Jagged-1 siRNA transfected PC-3 cells co-transfected with the luciferase construct resulted in a significantly decrease in relative luciferase activity, respectively, indicating that the decrease in CBF-1 binding was due to the inhibition of NICD (Supplementary Fig. 2A). The cell viability was further determined by MTT assay as shown by Figure 1B. We found that down-regulation of Notch-1 or Jagged-1 expression by siRNAs caused cell growth inhibition of PC-3 prostate cancer cell line. In order to further confirm the role of Notch on cell growth, the PC-3 cells were treated with gamma secretase inhibitors (GSI). We found that GSI inhibited the Notch-1 expression and consequently GSI had a strong effect in inhibiting the growth of PC-3 cell line (Fig. 1C). In addition, we have also tested the effects of down-regulation of Notch-1 or Jagged-1 on cell viability by clonogenic assay as shown below.

INHIBITION OF CELL GROWTH/SURVIVAL BY CLONOGENIC ASSAY

To determine the effect of Notch signaling on cell growth, cells were transfected with Notch-1 siRNA or Jagged-1 siRNA and assessed for cell viability by clonogenic assay. Both Notch-1 siRNA and Jagged-1 siRNA transfection resulted in a significant inhibition of colony formation of PC-3 cells when compared to control (Fig. 2A). Overall, the results from clonogenic assay was consistent with the MTT data

as shown in Figure 1B, suggesting that down-regulation of Notch-1 and Jagged-1 inhibited cell growth of PC-3 prostate cancer cells. Next, we examined whether the inhibition of cell growth was also accompanied by the induction of apoptosis in Notch-1 siRNA or Jagged-1 siRNA transfected cells.

DOWN-REGULATION OF NOTCH-1 AND JAGGED-1 EXPRESSION BY siRNA INDUCED APOPTOSIS

We investigated whether the overall growth inhibitory effects of Notch-1 siRNA or Jagged-1 siRNA are in part due to induction of apoptosis, which was examined by using an ELISA-based assay. These results provided convincing data that down-regulation of Notch-1 or Jegged-1 by siRNAs induced apoptosis in PC-3 prostate cancer cell line (Fig. 2B). These data suggest that the overall growth inhibitory activity of Notch-1 or Jagged-1 down-regulation was in part contributed by increased cell death.

DOWN-REGULATION OF NOTCH-1 INHIBITED AKT AND mTOR PATHWAY IN PC-3 CELLS

The cross-talk between Notch and Akt pathway has been previously reported in human cancer cell lines (Gutierrez and Look, 2007; Palomero et al., 2007; Bedogni et al., 2008). Therefore, we investigated whether Notch inactivation would reduce Akt function in PC-3 prostate cancer cell lines. We found that down-regulation of Notch-1 by siRNA or GSI led to decreased Akt phosphorylation and its downstream genes phosphorylation, such as mTOR, 4EBP-1, and S6K (Fig. 3A). To further confirm our results, we also did
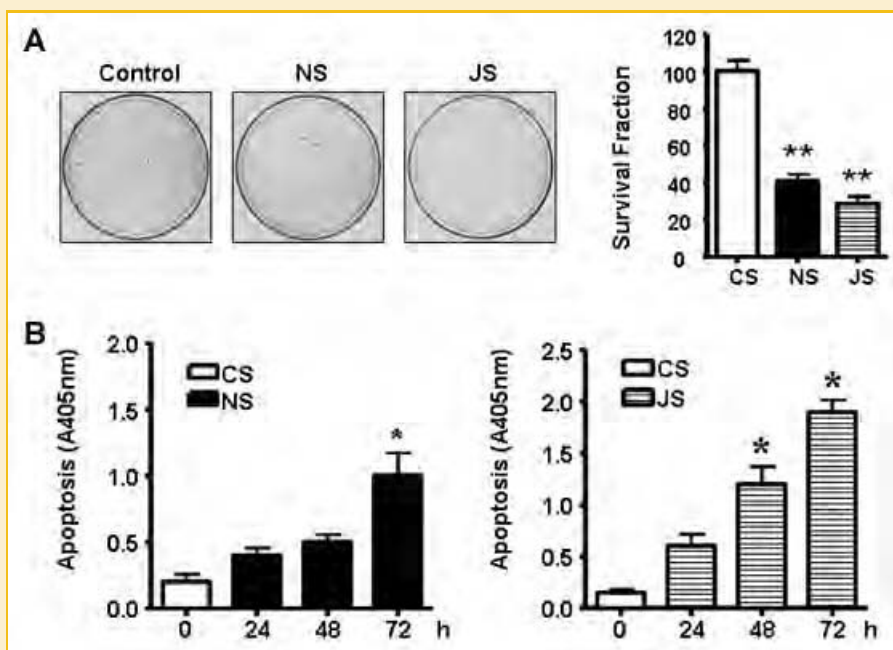

Fig. 2.   Effect of Notch-1 siRNA or Jagged-1 siRNA on PC-3 prostate cancer cell death. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. A: Left, cell survival of human PC-3 prostate cancer cell line. Notch-1 siRNA or Jagged-1 siRNA transfected PC-3 cells were evaluated by the clonogenic assay. Right, photomicrographic difference in colony formation in siRNA transfected cells compared to control. P values represent comparisons between cells transfected with siRNA and control using the paired t-test. B: Apoptosis was measured by Histone DNA ELISA in PC-3 cells transfected with Notch-1 siRNA or Jagged-1 siRNA for different periods of time as indicated in the figure. Values are reported as mean ± SD. *$P < 0.05$, compared to the control. We found that down-regulation of Notch-1 and Jagged-1 induced the apoptosis.
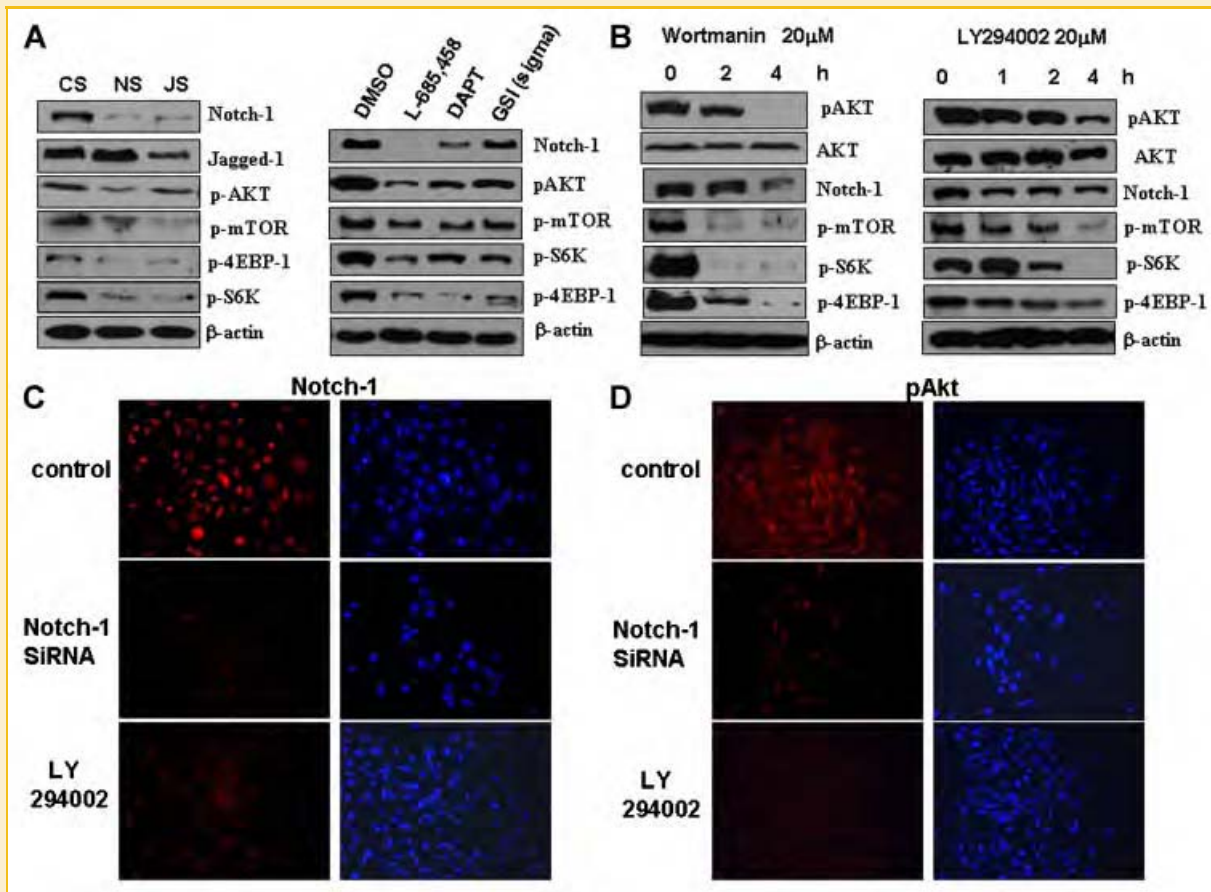
Fig. 3. Down-regulation of Notch-1 inhibited Akt and mTOR pathway in PC-3 cells. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. A: Down-regulation of Notch-1 by siRNA or GSI inhibited the activity of Akt and mTOR pathways as assessed by Western blot analysis. B: Inactivation of Akt by PI3K inhibitors (20 μM LY294002, 20 μM Wortmanin) inhibited the expression of Notch-1 and phospho-mTOR as assessed by Western blot analysis. C,D: Immunofluorescent staining showing lower levels of Notch-1 protein in the nucleus and pAkt in the cytoplasm and nucleus in the Notch-1 siRNA-transfected PC-3 cells. LY294002 eliminated Notch-1 expression and mTOR phosphorylation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

immunofluorescent staining. Indeed, we observed lower level of Notch-1 protein in the nucleus and pAkt in the cytoplasm and nucleus in the Notch-1 siRNA-transfected PC-3 cells (Fig. 3C,D). In contrast, up-regulation of Notch-1 by NICD transfection increased Akt phosphorylation and mTOR pathway (Supplementary Fig. 2B). It has been shown that Akt can control Notch-1 expression in melanoma (Bedogni et al., 2008), and thus we sought to determine whether Notch-1 expression could be controlled by Akt in PC-3 cells. We found that LY294002 and Wortmanin, the PI3K inhibitors, eliminated the expression of Notch-1, the phosphorylation of mTOR, S6K and 4EBP-1 (Fig. 3B), suggesting the existence of an interesting reciprocal regulation of Notch-1 and Akt pathways in prostate cancer cells. Moreover, we found that the expression of pAkt and its down-stream gene mTOR pathway was increased in Akt transfected PC-3 cells. Furthermore, inhibition of pAkt and mTOR pathway by Notch-1 siRNA was abrogated by Akt transfection (Supplementary Fig. 2C). Notch-1 could induce Akt signaling, but Notch-1 is also downstream of Akt and our results are consistent with previous findings in T-ALL cell lines and melanomas (Bedogni et al., 2008; Calzavara et al., 2008).

NOTCH-1 WAS DECREASED IN AKT KNOCK-OUT MEF CELL LINES

To further confirm our results showing that Notch-1 is a downstream effector of Akt, we examined the expression of Notch-1 in Akt WT, Akt-1KO, and Akt DKO MEF cell lines. We found that Notch-1 and Jagged-1 were decreased significantly in Akt DKO cell lines, which showed decreased pAkt and mTOR pathway (Fig. 4A,B). Moreover, Notch-1 siRNA and GSI decreased pAkt and p-mTOR in Akt WT cell lines. Further experiments were done where Akt WT and DKO MEFs were deprived of serum for 24 h and then stimulated by addition of 20% FBS, which showed that the Notch activity was impaired in Akt DKO cells (Fig. 4C), suggesting that many growth stimulating factors could increase Notch-1 activity through Akt signaling pathway.

DOWN-REGULATION OF NOTCH-1 OR JAGGED-1 BY SIRNA INHIBITED NF-κB DNA-BINDING ACTIVITY

We investigated whether the downstream effect of Notch-1 down-regulation was mechanistically associated with the NF-κB pathway in prostate cancer. Nuclear proteins from transfected cells were subjected to analysis for NF-κB p65 DNA-binding activity as measured by EMSA. The results showed that down-regulation of
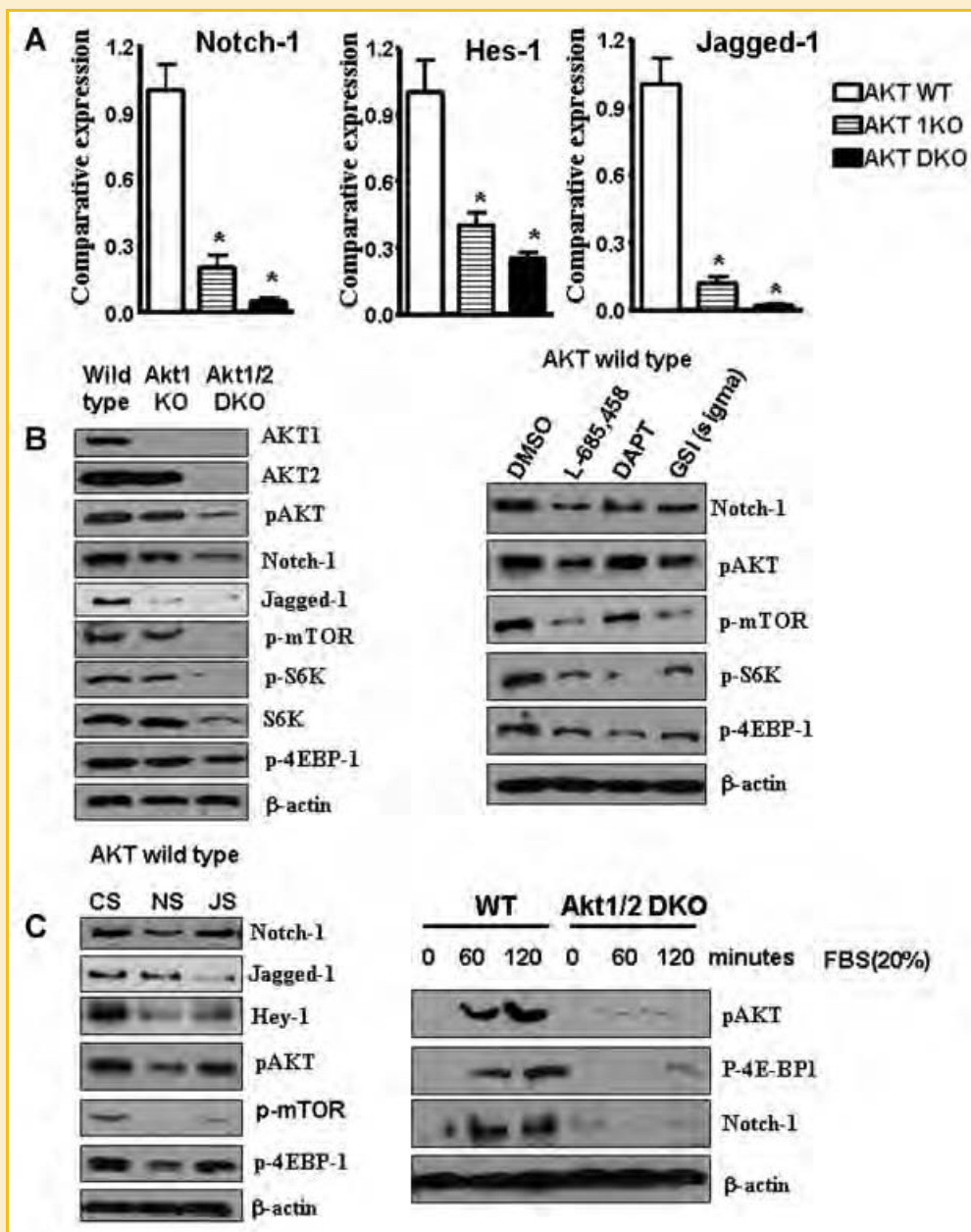
Fig. 4. Notch-1 was decreased in Akt knock-out MEF cell lines. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. A: Notch-1, Hes-1 and Jagged-1 mRNA were decreased significantly in Akt DKO cell lines using Real-time RT-PCR. B: Notch-1 and Jagged-1 proteins were decreased in Akt DKO cell lines using Western blot analysis. Notch-1 siRNA and GSI (L-685,458, DAPT) decreased pAkt and p-mTOR in Akt WT cell lines. C: Notch-1 siRNA and Jagged-1 siRNA decreased pAkt and p-mTOR in Akt WT cell lines using Western blot analysis. Akt WT and DKO MEFs were deprived of serum for 24 h and then stimulated by addition of 20% FBS for different time periods. We found that Notch activity was impaired in Akt DKO cells.

Notch-1 or Jagged-1 significantly inhibited NF-κB p65 DNA-binding activity compared to control (Fig. 5C). The effect of Notch-1 siRNA on p65 DNA-binding activity was not due to modulation of the p65 total protein level (Fig. 5B). Further, we found that Notch-1 siRNA or Jagged-1 siRNA reduced the basal levels of IKKα, IκBα and pIκBα (Fig. 5A). In contrast, up-regulation of Notch-1 activity by NICD transfection increased NF-κB p65 DNA-binding activity (data not shown). These results provided direct evidence in support of a mechanistic cross-talk between Notch-1 and NF-κB in prostate cancer. Furthermore, we also found that down-regulation of Notch-

1 or Jagged-1 inhibited NF-κB downstream gene expression, such as MMP-9, VEGF, and uPA (Fig. 5D).

DOWN-REGULATION OF NOTCH-1 OR JAGGED-1 DECREASED MMP-9, VEGF, AND uPA GENE TRANSCRIPTION AND THEIR ACTIVITIES

To explore whether Notch-1 or Jagged-1 siRNA transfection could decrease the transcription of MMP-9, VEGF, and uPA, real-time RT-PCR was employed. VEGF exists in at least six isoforms with variable amino acid residues produced through alternative splicing:
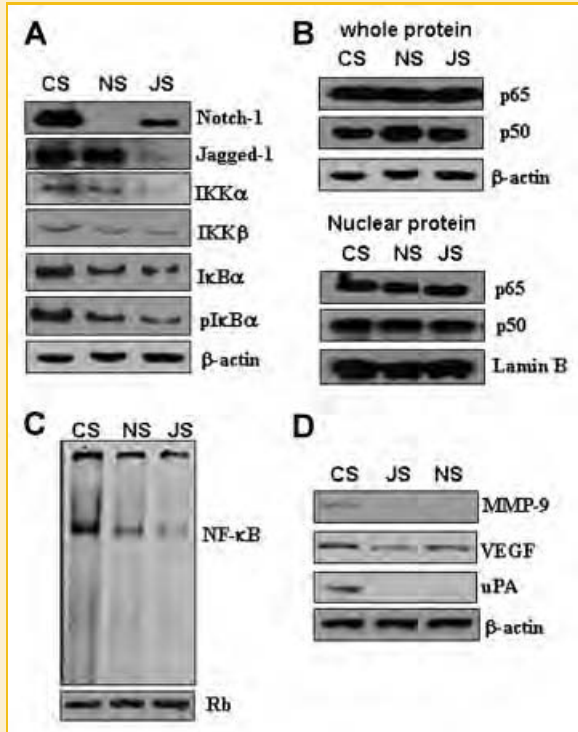
Fig. 5. Notch-1 siRNA and Jagged-1 siRNA inhibited the NF-κB DNA binding activity. A: Western blot analysis showed that Notch-1 siRNA and Jagged-1 siRNA inhibited the expression of IKKα, IκBα and pIκBα. B: Notch-1 siRNA and Jagged-1 siRNA did not change the protein expression of p65 and p50 using Western blot analysis. C: Notch-1 siRNA and Jagged-1 siRNA inhibited the NF-κB DNA binding activity in PC-3 cells as assessed by EMSA. D: Western blot analysis showed that Notch-1 siRNA and Jagged-1 siRNA inhibited the expression of MMP-9, VEGF, and uPA genes.

VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206. VEGF121, VEGF165, and VEGF189 are the major forms secreted by most cell types. Therefore, we detected the transcription of three major forms (VEGF121, 165, 189). We found that MMP-9, uPA, VEGF total mRNA, VEGF121, VEGF165, and VEGF189 were dramatically decreased in the siRNA transfected cells (Fig. 6A), which we believe is due to inactivation of NF-κB activity. Next, we examined whether the down-regulation of Notch-1 or Jagged-1 could also lead to a decrease in their activities in PC-3 prostate cancer cells. We found a marked decrease in the activity of MMP-9, uPA, and VEGF in Notch-1 and Jagged-1 siRNA transfected cells (Fig. 6B), which is also consistent with transcriptional inactivation of these genes.

DOWN-REGULATION OF NOTCH-1 OR JAGGED-1 DECREASED PROSTATE CANCER CELL MIGRATION AND INVASION

MMP-9, VEGF, and uPA are believed to be critically involved in the processes of tumor cell migration, invasion and metastasis. Since Notch-1 siRNA and Jagged-1 siRNA inhibited the expression and activity of MMP-9, VEGF, and uPA, we tested the effects of Notch-1 and Jagged-1 down-regulation on cancer cell migration and invasion. We found that down-regulation of Notch-1 or Jagged-1 decreased prostate cancer cell migration. Moreover, as illustrated in

Figure 6C, Notch-1 siRNA or Jagged-1 siRNA transfected cells showed a lower level of penetration through the matrigel-coated membrane compared with the control cells. The value of fluorescence from the invaded PC-3 prostate cancer cells was decreased about three- to fourfold compared with that of control cells (Fig. 6C), suggesting a direct role of Notch signaling in prostate cancer cell migration and invasion.

DISCUSSION

Aberrant expression of Notch pathway has been found in a variety of human cancers including breast, brain, cervical, lung, colon, head and neck, renal cell carcinoma, acute myeloid, Hodgkin and Large-cell lymphomas and pancreatic cancer (Miele, 2006; Miele et al., 2006). It has been reported that Notch-1 was over-expressed in prostate cancer cell lines and human prostate cancer tissues (Zayzafoon et al., 2004; Bin et al., 2009). Moreover, Notch-1 expression in human prostate cancer tissues increased with increasing tumor grade (Bin et al., 2009). However, the role of Notch pathway in prostate carcinogenesis remains poorly understood. Therefore, in the present study, we investigated the role of Notch-1 and Jagged-1 in cell proliferation in prostate cancer cells. In our study, down-regulation of Notch-1 and Jagged-1 elicited a dramatic effect on cell growth inhibition of PC-3 prostate cancer cells, as demonstrated by MTT assay and clonogenic assay. In addition, Notch-1 siRNA and Jagged-1 siRNA caused induction of apoptotic cell death, suggesting the growth inhibitory activity of Notch-1 or Jagged-1 down-regulation is in part attributed to an increase in cell death.

Hyperactivation of Akt pathway has previously been observed in human prostate cancer (de Souza et al., 2009). Recently, Notch has been shown to regulate the Akt pathway. Liu et al. (2006) have reported that Notch-1 activation enhanced melanoma cell survival and such effects of Notch signaling were mediated via activation of the Akt pathway. Palomero et al. (2008) found that Notch-1 induced up-regulation of the PI3K-Akt pathway via Hes-1, which negatively controls the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-ALL. Since Notch-1 has been reported to cross-talk with Akt pathway in human cancer cell lines (Gutierrez and Look, 2007; Bedogni et al., 2008; Meurette et al., 2009), we postulated whether cell growth inhibition was indeed due to the regulation of Akt pathway. We found that down-regulation of Notch-1 by siRNA or GSI decreased Akt phosphorylation. Interestingly, we also observed that inactivation of Akt by LY294002 and Wortmanin eliminated Notch-1 expression and mTOR phosphorylation. These results suggest the existence of an interesting reciprocal regulation of Notch-1 and Akt pathways. In other words Notch-1 could induce Akt signaling, but Notch-1 is also downstream of Akt pathway. Our results are consistent with previous findings in T-ALL cell lines, melanomas and breast cancer cells (Bedogni et al., 2008; Calzavara et al., 2008; Meurette et al., 2009).

The regulation mTOR activity by growth factors is mediated by the PI3K/Akt signaling pathway. The mTOR protein kinase has emerged as a critical player for controlling many cellular processes,
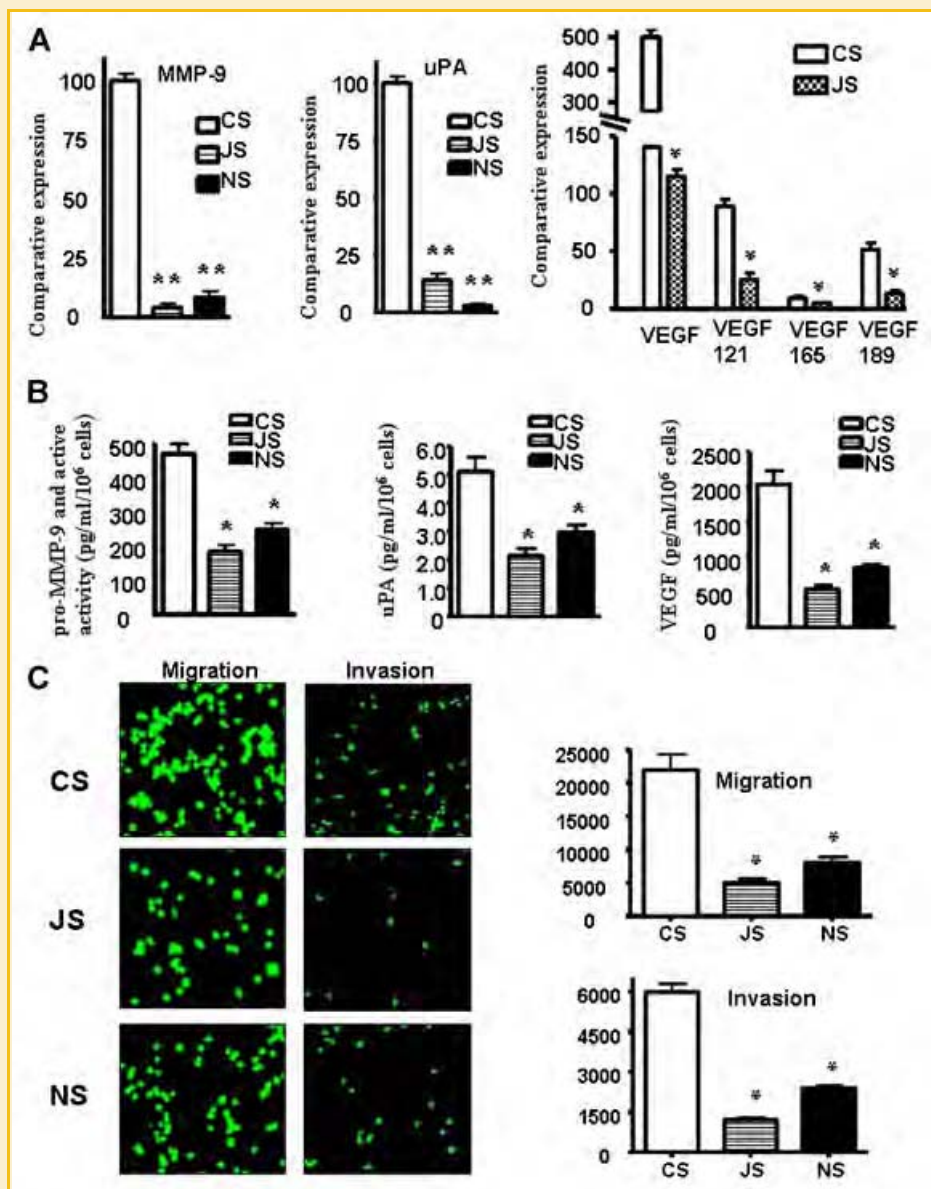
Fig. 6. Down-regulation of Notch-1 or Jagged-1 decreased prostate cancer cell migration and invasion. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. $^*P < 0.05$, $^{**}P < 0.01$ relative to control. A: Real-time RT-PCR showed that Notch-1 or Jagged-1 siRNA inhibited the expression of MMP-9, uPA, and VEGF genes at mRNA levels in PC-3 cells. B: Notch-1 siRNA or Jagged-1 siRNA inhibited the activity of MMP-9, uPA, and VEGF in PC-3 cells as assessed by ELISA. C: Left; Notch-1 siRNA or Jagged-1 siRNA decreased PC-3 prostate cancer cell migration and invasion. Right; values of fluorescence from the migrated cells or invaded cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

such as cell growth and cell division by receiving stimulatory signals from Notch and Akt (Mungamuri et al., 2006; Chan et al., 2007). It has been reported that inhibition of tumor suppressor protein p53 by ICN mainly occurs through mTOR using the PI3K/Akt pathway, as rapamycin treatment abrogated ICN inhibition of tumor suppressor protein p53 and reversed the chemo-resistance of breast cancer and T-ALL (Mungamuri et al., 2006). It is well known that mTOR regulates translation rates and cell proliferation in part by phosphorylating two major targets, 4E-BP1 and ribosomal protein S6 kinases (S6K1 and S6K2). In deed, we found that down-regulation of Notch-1 by siRNA or GSI decreased mTOR phosphorylation and its target gene 4EBP-1 and S6K phosphorylation. We also found that

inactivation of Akt by LY294002 and Wortmanin eliminated mTOR and its target 4EBP-1, s6K phosphorylation.

Akt regulates a number of downstream effectors including NF-κB. NF-κB has been reported to cross-talk with Notch pathway. The cross-talk between NF-κB and Notch is extraordinarily complex. Constitutive levels of Notch activity are essential to maintain NF-κB activity in various cell types. Levels of basal and stimulation-induced NF-κB activity were significantly decreased in mice with reduced Notch levels (Osipo et al., 2008). Recently, Song et al. (2008) reported that Notch-1 stimulated NF-κB activity in cervical cancer cells by associating with the IKK signalosome through IKKα. NF-κB was also previously shown to increase Notch-1 activity indirectly by

increasing the expression of Jagged-1 in lymphoma and myeloma cells (Bash et al., 1999). These reports suggest that, at least, in some contexts stimuli that activate NF-κB could lead to Notch activation, and conversely inactivation of Notch could inhibit NF-κB. In our study, we found that down-regulation of Notch inhibited the NF-κB DNA binding activity. Moreover, we found that Notch-1 siRNA or Jagged-1 siRNA reduced the basal levels of IKKα, IκBα, and pIκBα, which are consistent with previous findings in cervical cancer cells (Song et al., 2008). However, further in-depth molecular mechanistic studies are required in order to determine how Notch-1 crosstalk with NF-κB.

It is well accepted that many important molecules, such as MMP-9, VEGF, uPA, are involved in tumor cell invasion and metastasis. Since we found that down-regulation of Notch-1 and Jagged-1 inhibited the expression and activities of NF-κB downstream genes such as, MMP-9, VEGF, and uPA, we tested the effects of down-regulation of Notch-1 and Jagged-1 on the migration and invasion of prostate cancer cells. We found that down-regulation of Notch-1 or Jagged-1 inhibited migration and invasion of prostate cancer cells through matrigel, and these results are consistent with previous findings of Bin et al. (2009). Our results also suggest that down-regulation of Notch-1 or Jagged-1 could inhibit cancer cell migration and invasion, which was in part due to down-regulation of NF-κB and its downstream target genes such as MMP-9, uPA, and VEGF.

In summary, we presented experimental evidence which strongly suggest the role of Notch-1 down-regulation as a potential anti-tumor and anti-metastatic approach in prostate cancer. Down-regulation of Notch-1 or Jagged-1 inhibited cell growth with reduced Akt phosphorylation, its downstream gene mTOR phosphorylation and inactivated NF-κB signaling. From these results, we conclude that down-regulation of Notch-1 or Jagged-1 could potentially be an effective therapeutic approach for the inactivation MMP-9, uPA, and VEGF, which is likely to result in the inhibition of cell growth, migration, invasion and metastasis of prostate cancer. On the basis of our results, we propose a hypothetical pathway by which Jagged-1 and Notch-1 inactivation may induce apoptotic cell death and inhibit invasion of PC-3 cells, which is in part mediated via dysregulation of Akt, mTOR, and NF-κB signaling pathway (Supplemental Fig. 2). Taken together, these data provide a rationale for targeting the Notch signaling pathways for the treatment of prostate cancer in the future.

ACKNOWLEDGMENTS

REFERENCES

Bash J, Zong WX, Banga S, Rivera A, Ballard DW, Ron Y, Gelinas C. 1999. Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. EMBO J 18:2803–2811.

Bedogni B, Warneke JA, Nickoloff BJ, Giaccia AJ, Powell MB. 2008. Notch1 is an effector of Akt and hypoxia in melanoma development. J Clin Invest 118:3660–3670.

Bin HB, Adhami VM, Asim M, Siddiqui IA, Bhat KM, Zhong W, Saleem M, Din M, Setaluri V, Mukhtar H. 2009. Targeted knockdown of Notch1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. Clin Cancer Res 15:452–459.

Calzavara E, Chiaramonte R, Cesana D, Basile A, Sherbet GV, Comi P. 2008. Reciprocal regulation of Notch and PI3K/Akt signalling in T-ALL cells in vitro. J Cell Biochem 103:1405–1412.

Chan SM, Weng AP, Tibshirani R, Aster JC, Utz PJ. 2007. Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. Blood 110:278–286.

de Souza PL, Russell PJ, Kearsley J. 2009. Role of the Akt pathway in prostate cancer. Curr Cancer Drug Targets 9:163–175.

Gutierrez A, Look AT. 2007. NOTCH and PI3K-AKT pathways intertwined. Cancer Cell 12:411–413.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. 2009. Cancer Statistics, 2009. CA Cancer J Clin 59: 225–249.

Kopan R, Ilagan MX. 2009. The canonical Notch signaling pathway: Unfolding the activation mechanism. Cell 137:216–233.

Leong KG, Gao WQ. 2008. The Notch pathway in prostate development and cancer. Differentiation 76:699–716.

Liu ZJ, Xiao M, Balint K, Smalley KS, Brafford P, Qiu R, Pinnix CC, Li X, Herlyn M. 2006. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. Cancer Res 66:4182–4190.

Martin DB, Gifford DR, Wright ME, Keller A, Yi E, Goodlett DR, Aebersold R, Nelson PS. 2004. Quantitative proteomic analysis of proteins released by neoplastic prostate epithelium. Cancer Res 64:347–355.

Meurette O, Stylianou S, Rock R, Collu GM, Gilmore AP, Brennan K. 2009. Notch activation induces Akt signaling via an autocrine loop to prevent apoptosis in breast epithelial cells. Cancer Res 69:5015–5022.

Miele L. 2006. Notch signaling. Clin Cancer Res 12:1074–1079.

Miele L, Osborne B. 1999. Arbiter of differentiation and death: Notch signaling meets apoptosis. J Cell Physiol 181:393–409.

Miele L, Miao H, Nickoloff BJ. 2006. Notch signaling as a novel cancer therapeutic target. Curr Cancer Drug Targets 6:313–323.

Mungamuri SK, Yang X, Thor AD, Somasundaram K. 2006. Survival signaling by Notch1: Mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Res 66:4715–4724.

Osipo C, Golde TE, Osborne BA, Miele LA. 2008. Off the beaten pathway: The complex cross talk between Notch and NF-kappaB. Lab Invest 88: 11–17.

Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, Caparros E, Buteau J, Brown K, Perkins SL, Bhagat G, Agarwal AM, Basso G, Castillo M, Nagase S, Cordon-Cardo C, Parsons R, Zuniga-Pflucker JC, Dominguez M, Ferrando AA. 2007. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med 13:1203–1210.

Palomero T, Dominguez M, Ferrando AA. 2008. The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. Cell Cycle 7:965–970.

Rizzo P, Osipo C, Foreman K, Golde T, Osborne B, Miele L. 2008. Rational targeting of Notch signaling in cancer. Oncogene 27:5124–5131.

Santagata S, Demichelis F, Riva A, Varambally S, Hofer MD, Kutok JL, Kim R, Tang J, Montie JE, Chinnaiyan AM, Rubin MA, Aster JC. 2004. JAGGED1 expression is associated with prostate cancer metastasis and recurrence. Cancer Res 64:6854–6857.

Shou J, Ross S, Koeppen H, de Sauvage FJ, Gao WQ. 2001. Dynamics of notch expression during murine prostate development and tumorigenesis. Cancer Res 61:7291–7297.

Skeen JE, Bhaskar PT, Chen CC, Chen WS, Peng XD, Nogueira V, Hahn-Windgassen A, Kiyokawa H, Hay N. 2006. Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell 10:269–280.

Song LL, Peng Y, Yun J, Rizzo P, Chaturvedi V, Weijzen S, Kast WM, Stone PJ, Santos L, Loredo A, Lendahl U, Sonenshein G, Osborne B, Qin JZ, Pannuti A, Nickoloff BJ, Miele L. 2008. Notch-1 associates with IKKalpha and regulates IKK activity in cervical cancer cells. Oncogene 27:5833–5844.

Villaronga MA, Bevan CL, Belandia B. 2008. Notch signaling: A potential therapeutic target in prostate cancer. Curr Cancer Drug Targets 8:566–580.

Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y, Sarkar FH. 2006a. Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{kappa}B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 66:2778–2784.

Wang Z, Zhang Y, Li Y, Banerjee S, Liao J, Sarkar FH. 2006b. Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. Mol Cancer Ther 5:483–493.

Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH. 2007. Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer Res 67:8293–8300.

Wang Z, Li Y, Banerjee S, Sarkar FH. 2008. Exploitation of the Notch signaling pathway as a novel target for cancer therapy. Anticancer Res 28:3621–3630.

Zayzafoon M, Abdulkadir SA, McDonald JM. 2004. Notch signaling and ERK activation are important for the osteomimetic properties of prostate cancer bone metastatic cell lines. J Biol Chem 279:3662–3670.

Zhang Y, Wang Z, Ahmed F, Banerjee S, Li Y, Sarkar FH. 2006. Down-regulation of Jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells. Int J Cancer 119:2071–2077.

Review

Exploitation of the Notch Signaling Pathway as a Novel Target for Cancer Therapy

ZHIWEI WANG, YIWEI LI, SANJEEV BANERJEE and FAZLUL H. SARKAR

*Department of Pathology, Karmanos Cancer Institute, Wayne State
University School of Medicine, Detroit, Michigan, U.S.A.*

Abstract. *The Notch signaling pathway appears to be
responsible for maintaining a balance between cell
proliferation and apoptosis and thus it has been suggested that
Notch may play an important role in species development and
in the development and progression of several malignancies.
Therefore, the Notch signaling pathway may represent a novel
therapeutic target, which could have the highest therapeutic
impact in modern medicine. This review describes the
mechanisms of signal transduction of the Notch signaling
pathway and provides emerging evidence in support of its role
in the development of human malignancies. Further attempts
have been made to summarize the role of several
chemopreventive agents that could be useful for targeted
inactivation of Notch signaling, which could become a novel
approach for cancer prevention and treatment.*

Notch signaling is involved in cell proliferation and apoptosis
which affects the development and function of many organs.
Notch genes encode proteins which can be activated by
interacting with a family of its ligands. To date, four vertebrate
Notch genes have been identified: *Notch-1-4*. In addition, five
ligands, Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-
like 4), Jagged-1 and Jagged-2, have been found in mammals
(1, 2). Although these four Notch receptors show subtle
differences in their extracellular and cytoplasmic domains, they
are very similar. The extracellular domain of Notch possesses
many epidermal growth factor (EGF)-like repeats, which
participate in ligand binding. The amino-terminal EGF-like
repeats are followed by cysteine-rich Notch Lin12 repeats

Correspondence to: Fazlul H. Sarkar, Ph.D., Department of
Pathology, Karmanos Cancer Institute, Wayne State University
School of Medicine, 9374 Scott Hall, 540 E Canfield, Detroit, MI
48201 U.S.A. Tel: +1 3135768327, Fax: +1 3135768389, e-mail:
sarkarf@karmanos.org

Key Words: Notch, cancer, signal pathway, review.

(N/Lin12) that prevent signaling in the absence of the ligand.
The cytoplasmic region of Notch conveys the signal to the
nucleus; it contains a recombination signal-binding protein 1 for
J-kappa (RBP-J)-association molecule (RAM) domain, ankyrin
repeats, nuclear localization signals (NLS), a trans-activation
domain (TAD) and a region rich in proline, glutamine, serine
and threonine residues (PEST) sequence (3).

Notch signaling is initiated by a receptor-ligand interaction
between two neighboring cells. Upon activation, Notch is
cleaved, releasing the intracellular domain of the Notch (ICN)
through a cascade of proteolytic cleavages by the
metalloprotease tumor necrosis factor-α-converting enzyme
(TACE) and γ-secretase. The first cleavage is mediated by
TACE, which cleaves the receptor in the extracellular domain.
The released extracellular domain is then trans-endocytosed by
the ligand-expressing cell. The second cleavage caused by the
γ-secretase activity of a multi-protein complex consisting of
presenilin, nicastrin, *etc.* releases the ICN which is then ready to
be translocated into the nucleus for transcriptional activation of
Notch target genes (1, 2, 4). Therefore, inhibiting γ-secretase
function prevents the cleavage of the Notch receptor, blocking
Notch signal transduction. In the absence of ICN cleavage,
transcription of Notch target genes is inhibited by a repressor
complex mediated by the CSL (CBF1, suppressor of hairless,
Lag-1). When ICN enters the nucleus, it recruits transcription
activators to the CSL complex and converts it from a
transcriptional repressor into an activator, which activates the
Notch target genes (1, 2). A few Notch target genes have been
identified, some of which are dependent on Notch signaling in
multiple tissues, while others are tissue specific. Notch target
genes include the *Hes-1* (hairy enhance of split-1), *nuclear
factor-kappa B* (NF-κB), *cyclin D1* and *c-myc* (1, 2, 5).

Notch Signaling in Cancer

Notch signaling plays important roles in maintaining the
balance between cell proliferation, differentiation and
apoptosis (1). The *Notch* gene is abnormally activated in

many human malignancies. It has been reported that the function of Notch signaling in tumorigenesis can be either oncogenic or antiproliferative, and the function is context dependent (1, 6). In a limited number of tumor types, including human hepatocellular carcinoma and small cell lung cancer, Notch signaling is antiproliferative rather than oncogenic (7-9). However, most of the studies have shown an opposite function of Notch in many human carcinomas, including pancreatic cancer (1, 2, 8-11). It has been reported that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer (8-15).

Moreover, high-level expression of Notch-1 and its ligand Jagged-1 is associated with poor prognosis in breast and prostate cancer. Specifically, patients with tumors expressing high levels of Jagged-1 or Notch-1 had a significantly poorer overall survival compared with patients expressing low levels of these genes. Moreover, a synergistic effect of high-level Jagged-1 and high-level Notch-1 co-expression on overall survival was observed (16). Notch-1 is an important prognostic marker in T-cell acute lymphoblastic leukemia (T-ALL) and its predictive value could be even further increased if co-evaluated with other T-cell-related regulatory genes (17). Jagged-1 is highly expressed in metastatic prostate cancer as compared with localized prostate cancer or benign prostatic tissues. Furthermore, high Jagged-1 expression in a subset of clinically localized tumors was significantly associated with recurrence, suggesting that Jagged-1 may be a useful marker in distinguishing indolent vs. aggressive prostate carcinomas (18). Multiple oncogenic pathways, such as NF-κB, Akt, Sonic hedgehog (Shh), mammalian target of rapamycin (mTOR), Ras, Wnt, epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) signaling have been reported to cross-talk with the Notch pathway and thus it is believed that the cross-talk between Notch and other signaling pathways plays an important role in tumor aggressiveness.

Notch and NF-κB Signaling

The molecular mechanism(s) by which Notch signaling induces tumor growth has not been fully elucidated. Notch-1 has been reported to cross-talk with another major cell growth and apoptotic regulatory pathway, namely NF-κB. NF-κB plays important roles in the control of cell growth, differentiation, apoptosis, and inflammation (19, 20). NF-κB mediates survival signals that inhibit apoptosis and promote cancer cell growth. The activation of NF-κB involves the phosphorylation of IκB (I-kappa-B), an inhibitory binding partner of the NF-κB complex, for ubiquitination and degradation through the proteasome degradation pathway.

This allows the translocation of NF-κB into the nucleus where it activates the transcription of genes. A key regulatory step in the NF-κB pathway is the activation of a high molecular weight IKK (IκB kinase) complex in which catalysis is thought to be via kinases, including IKKα and IKKβ, which directly phosphorylate IκB proteins (20).

Notch-1 has been reported to strongly induce NF-κB2 promoter activity in reporter assays (21) and to induce the expression of several NF-κB subunits (22). Notch ligands activate NF-κB in human keratinocytes and the down-regulation of Notch-1 results in lower NF-κB activity. The levels of basal and stimulation-induced NF-κB activity were found to be significantly lower in mice with reduced Notch levels (23, 24). Vilimas et al. examined a murine model of T-ALL induced by overexpression of ICN and found that the NF-κB target genes were up-regulated in the ICN-transformed cells. In these cells, and in human cell lines derived from spontaneous T-ALL, ICN interacts with the IKK signalosome, increasing its IκBα kinase activity (25). It has recently been discovered that in colorectal carcinoma cells, nuclear IKKα phosphorylates SMRT (silencing mediator of retinoid and thyroid hormone receptor) not only in association with NF-κB but also in association with CSL (26). Constitutive levels of Notch activity are essential to maintain NF-κB activity in various cell types. Indeed, we have found that down-regulation of Notch-1 reduced NF-κB activity. In contrast, overexpression of wild-type Notch-1 cDNA enhanced NF-κB activity (27). We also found that down-regulation of Notch-1 caused attenuation of NF-κB consistent with the down-regulation of NF-κB downstream genes such as *vascular endothelial growth factor (VEGF)* and *matrix metalloproteinase-9 (MMP-9)*, resulting in the inhibition of cancer cell invasion through matrigel (27).

However, several groups have reported that NF-κB can also regulate Notch expression (28, 29). The observations reported in the literature so far offer a complex and incomplete picture of the interactions between these two key cell fate-determining pathways. As is becoming increasingly clear in the case of other pathways, these interactions can be cooperative or antagonistic and multiple levels of feedback are possible depending on the context. The physiological relevance of these interactions needs to be thoroughly investigated. However, it can be safely stated that those planning to manipulate the Notch-signaling pathway for experimental or therapeutic purposes would do very well to examine the possible effects on NF-κB signaling pathway and *vice versa* (30).

Notch and Akt Signaling

Akt (also known as protein kinase B) is an evolutionarily conserved serine/threonine kinase. Three isoforms, Akt1, Akt2 and Akt3, are expressed in mammals. Akt is activated

by phosphatidylinositol 3-kinase (PI3K), which transmits signals from cytokines, growth factors and oncoproteins to multiple targets, including Akt. Activation of PI3K localizes Akt to the plasma membrane *via* the pleckstrin homology domain of Akt, where Akt is activated by phosphorylation at Thr[308] and Ser[473] (31). Akt plays a critical role in mammalian cell survival signaling and has been shown to be activated in various malignancies (31-33). Activated Akt functions to promote cell survival by inhibiting apoptosis through inactivation of several pro-apoptotic factors including Bcl-xL/Bcl-2-associated death (BAD), forkhead transcription factors and caspase-9 (34-36). Several studies have also shown that Akt regulates the NF-κB pathway *via* the phosphorylation and activation of molecules in the NF-κB pathway (37, 38).

Recently, Notch has been shown to regulate the Akt pathway. Liu *et al*. have reported that Notch-1 activation enhanced melanoma cell survival and such effects of Notch signaling were mediated by activation of the Akt pathway and the mitogen-activated protein kinase (MAPK) pathway (39). Palomero *et al*. found that Notch-1 induced up-regulation of the PI3K-Akt pathway *via* Hes-1, which negatively controls the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-ALL. The loss of PTEN and constitutive activation of Akt in T-ALL induced increased glucose metabolism and bypassed the requirement of Notch-1 signaling to sustain cell growth (40). Moreover, Palomero *et al*. identified loss of PTEN as a critical event leading to resistance to Notch inhibition, which caused the transfer of the phenomenon of "oncogene addiction" from the Notch-1 signaling to the PI3K/Akt signaling pathway (41, 42). Emerging evidence suggests that expression of the Notch ligand Jagged in human keratinocytes and cervical cancer cell lines leads to Akt phosphorylation and induction of a frank PI3K-dependent epithelial-mesenchymal transition (EMT) phenotype characterized by enhanced motility, morphological changes, E-cadherin down-regulation and up-regulation of vimentin and fibronectin (43). These observations also suggest that there is an urgent need for simultaneous inhibition of both pathways as a means of improving therapeutic efficacy for the treatment of most human malignancies.

Notch and mTOR Signaling

The mammalian target of rapamycin (mTOR) pathway has been reported to cross-talk with the Notch pathway (44-46). mTOR regulates translation rates and cell proliferation in part by phosphorylating two major targets, the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinases (S6K1 and S6K2). Upon phosphorylation, 4E-BP1 is released from eIF4E, allowing eIF4E to assemble with other translation

initiation factors to initiate cap-dependent translation. The eIF4E is thought to enhance the translation of transcripts possessing either complex 5'-untranslated region secondary structure and/or *via* upstream open reading frames, which often encode proteins associated with a proliferative response. S6K1 directly phosphorylates the 40S ribosomal protein S6, and then promotes ribosome biogenesis (47). mTOR exists in two distinct complexes, mTORC1 and mTORC2, within the cells: mTORC1 consists of mTOR, G-protein β-subunit-like protein (GβL), raptor and proline-rich Akt substrate of 40 kiloDaltons (PRAS40); and mTORC2 contains mTOR, GβL, rictor and stress-activated protein kinase-interacting protein 1 (SIN1). The raptor-containing complex is sensitive to rapamycin and regulates cell growth and proliferation in part through phosphorylating S6K and 4E-BP1. The rictor-containing complex is not sensitive to rapamycin (48).

The mTOR protein kinase has emerged as a critical player for controlling many cellular processes, such as cell growth and cell division, by receiving stimulatory signals from Notch and PI3K (44-46). Notch receptor activation induces the expression of specific target genes *Hes-3* and *Shh* through rapid activation of cytoplasmic signals, including Akt, mTOR and signal transducer and activator of transcription 3 (STAT3), and thereby promotes the survival of neural stem cells (44). Inhibition of tumor protein p53 by ICN mainly occurs through mTOR using the PI3K/Akt pathway, as rapamycin treatment abrogated ICN inhibition of tumor protein p53 and reversed the chemoresistance in breast cancer and T-ALL (46). Further, ectopic expression of eIF4E inhibited p53-induced apoptosis and conferred protection against p53-mediated cytotoxicity to a similar extent as that of ICN overexpression, but it was not reversed by rapamycin, which indicated that eIF4E is the major target of mTOR in Notch-1-mediated survival signaling (46). Recently, Chan *et al*. reported that the mTOR pathway is positively regulated by Notch in T-ALL cells. They found that the effect of gamma secretase inhibitor (GSI) on the mTOR pathway was independent of changes in PI3K and Akt activity, but was rescued by expression of c-Myc, a direct transcriptional target of Notch, implicating c-Myc as an intermediary between Notch and mTOR (45). Moreover, T-ALL cell growth was suppressed in a highly synergistic manner by simultaneous treatment with the mTOR inhibitor rapamycin and GSI, which represents a rational drug combination for treating this aggressive human malignancy (45).

Notch and EGFR Signaling

EGFR is a transmembrane tyrosine kinase protein. After ligand binding, EGFR dimerizes, either as a homodimer or heterodimer with other members of the EGFR family. EGFR is then auto-phosphorylated or trans-phosphorylated at

specific tyrosine residues for its activation, resulting in the activation of multiple downstream signaling cascades, including PI3K/Akt, and extracellular signal-regulated kinase (ERK), ultimately leading to increased cellular proliferation and the prevention of programmed cell death. Therefore, excessive activation of EGFR-dependent pathways may have an important role in the biological aggressiveness of human cancer.

It has been reported that Notch-1 inhibition reduced *EGFR* mRNA and EGFR protein in glioma and other cell lines, whereas transfection with Notch-1 increased EGFR expression. Additionally, a significant correlation in levels of *EGFR* and *Notch-1* mRNA in primary high-grade human gliomas has been found. Subsequent experiments have shown that p53, an activator of the *EGFR* promoter, is regulated by Notch-1. These results showed that Notch-1 up-regulates EGFR expression and also demonstrated Notch-1 mediated up-regulation of p53 in gliomas (49). Recently, Zhang *et al.* demonstrated that γ-secretase regulates EGFR through releasing ICN generation, which directly binds to the *EGFR* promoter and regulates *EGFR* gene expression (50). The findings from our laboratory have shown that the EGFR inhibitor caused marked inhibition of pancreatic cancer cell growth, which was accompanied by increased apoptosis and concomitant attenuation of the Notch-1 signaling pathway (51). These results provide some clear evidence in support of an interactive role of Notch with EGFR signaling in human cancer.

Notch and PDGF Signaling

Many tumors have been shown to overexpress the PDGF family members (52-54). The PDGFs are composed of four different polypeptide chains encoded by different genes. Four PDGF family members have been identified to date, PDGF A-D. The four PDGF chains assemble into disulphide-bonded dimers *via* homo- or heterodimerization, and five different dimeric isoforms have been described to date, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. It is notable that no heterodimers involving PDGF-C and PDGF-D chains have been described (54, 55). The PDGFs have a common structure with the typical growth factor domain involved in the dimerization of the two subunits and in receptor binding and activation. PDGF-A and PDGF-B have short *N*-terminal extensions that undergo intracellular proteolytic processing for activation, while both PDGF-C and PDGF-D chains display a distinct protein domain, the so called complement C1r/C1s, Uegf, Bmp1 (CUB) domain, as part of their *N*-terminal extensions. Several reports have indicated that the CUB domain of PDGF-D has to be cleaved extracellularly to make the COOH-terminal growth factor domain active for PDGF-D binding to its cognate receptor (54, 55). PDGFs exert their

cellular effects by activating two structurally related receptor tyrosine kinases, PDGFR-α and PDGFR-β. The PDGF-A, PDGF-B and PDGF-C are secreted as homodimers or heterodimers and bind to dimeric PDGF receptors (PDGFR) composed of α- and/or β-chains, while PDGF-D specifically binds to and activates its cognate receptor PDGFR-β (56). It has been reported that PDGF signaling regulates the expression of the Notch-1 receptor in some cell lines (57). The expression of PDGF-B correlates with Notch ligand Dll-4 expression in developing retinal arteries (58). We also found that down-regulation of PDGF-D leads to the inactivation of Notch-1 and NF-κB DNA-binding activity and, in turn, down-regulates the expression of its target genes, such as *VEGF* and *MMP-9*. Therefore, the inactivation of PDGF-D-mediated cell invasion and angiogenesis that we have reported could in part be due to inactivation of Notch-1 activity (53). These results further suggest that the combination of the inhibitors of PDGF and Notch signaling could be therapeutically useful and thus further studies are warranted in this area.

Notch and Sonic Hedgehog Signaling

The hedgehog family of growth factors activates a highly conserved signaling system for cell–cell communication that regulates cell proliferation and differentiation during development. Abnormal activation of the hedgehog pathway has been demonstrated in a variety of human tumors, including those of the skin, brain, lung and digestive tract. The hedgehog family of signaling proteins consists of secreted proteins that signal through both autocrine and paracrine mechanisms to control cell proliferation, differentiation and morphology. There are three known hedgehog ligands, Sonic (Shh), Indian (Ihh) and Desert (Dhh). The Shh is more closely related to Ihh, while Dhh is more closely related to the hedgehog of *Drosophila*. The hedgehog proteins exert their function by binding to a 12-pass transmembrane protein called patched (PTCH) (59, 60). This interaction relieves the inhibitory effect of PTCH on a serpentine protein called smoothened (SMO). The SMO is then hyperphosphorylated and has been recently shown to localize to primary cilia. This pathway ultimately concludes with the activation and repression of target genes through the Gli family of transcription factors. In mammals, there are three Gli transcription factors (Gli-1, -2, -3) that regulate the transcription of target genes (60).

Cross-talk also exists between the Notch pathway and both Wnt and Shh signaling. Notch-1 normally represses Wnt and Shh signaling, both of which are known to regulate tumorigenesis. In Notch-1 null skin, activation of Wnt and Shh pathways resulted in the development of basal cell carcinoma and squamous cell carcinoma in the mouse (61). Katoh reported that hedgehog signals result in the up-regulation of the *Jagged-2* gene, which activates Notch

signaling (62). Moreover, Hes-1, a principal downstream target of the Notch pathway, was found to be a target of Shh in mesodermal and neural cells (59). Medulloblastoma arising in heterozygous *PTCH* knockout mice displays an elevated expression of a number of Notch pathway genes, as do similar tumors arising in mice expressing an oncogenic form of *Smo* (63-65).

The Wnt signal transduction pathway also plays an important role during embryonic development, regulating cell proliferation and survival of immature cells. However, its improper function can lead to harmful consequences for humans, such as aberrant cell proliferation and therefore, cancer. The human *Wnt* gene family consists of 19 members, which regulate Wnt glycoproteins. The classical view of this pathway is that, upon binding to their receptors, Wnt proteins induce intracellular inactivation of glycogen synthase kinase-3β (GSK-3β), a component of the destruction complex, which also contains adenomatous polyposis coli (APC) and axin (66). This process results in the dephosphorylation and stabilization of β-catenin, a substrate of GSK-3β, which leads to the nuclear translocation of β-catenin. In the nucleus, β-catenin acts as a transcriptional co-activator and activates genes involved in cell proliferation and survival (66).

Several recent studies have suggested clear links between Wnt and Notch signaling. Squamous cell carcinoma develops spontaneously in the epidermis of mice expressing a dominant negative form of Mastermind1, which functions as an adaptor for RBP-J dependent Notch signaling. Dominant negative Mastermind1 inhibits Notch signaling, resulting in the activation of the Wnt pathway in keratinocytes (67). Enhanced Notch signaling activity has been observed in multiple intestinal neoplasia mice, which display hyperactivation of the Wnt signal because of a mutation of the *APC* gene, suggesting that the Wnt signal is mechanistically epistatic to the Notch signal (68). Recently, an intriguing report presented evidence that Notch and Wnt pathways act in synergy to maintain the hematopoietic stem cell (HSC) pool (69). These findings suggest that Wnt and Notch signaling together could play a role in self-renewal of HSCs. Moreover, observations that Wnt3a regulates the expression of established Notch target genes and that inhibition of the Wnt signaling component of GSK-3 affects HSC fate options through mechanisms involving regulation of both Wnt and Notch target genes suggest that the two pathways belong to a network of regulatory circuits controlling the HSC pool (69-71).

Notch and Ras

Ras is a small GTPase that cycles between an inactive, GDP-bound state and an active, GTP-bound state. In mammals, Ras appears to be a central player in multiple signaling pathways. Ras can be activated by a wide variety of upstream signals, and Ras-GTP can bind and activate multiple downstream targets (72). Ras acts primarily within a receptor tyrosine kinase (RTK)–Ras–MAPK/ERK pathway. Among many RTKs, EGFR is one major RTK that signals through Ras and ERK (72).

Stockhausen *et al*. have shown that transforming growth factor (TGFα), a known activator of RTKs and Ras signaling, can drive cell proliferation and at the same time Ras activation could induce the expression of the Notch target Hes-1 in a neuroblastoma cell line. These studies have shown that Hes-1 expression was induced simultaneously with increased ERK1/2 phosphorylation in TGFα-stimulated neuroblastoma cells, suggesting Hes-1, a key mediator of Notch signaling, can be regulated by the Ras/MAPK signaling pathway (73). In addition to Notch being a mediator of Ras signaling, there is also some evidence for Ras as an effector of Notch. Fitzgerald *et al*. have shown that transformation by Notch-4 required active Ras signaling, in particular the activity of ERK and PI3K (74). For example, in human cultured cells transformed by a combination of active Ras, SV40 and human telomerase reverse transcriptase (hTERT), Ras acts through p38 MAPK to up-regulate the expression of Dll-1 and Notch-1. Interfering with Notch signaling in this system inhibited anchorage-independent growth, suggesting that sequential signaling through Notch is critical for Ras-induced transformation (75). Similarly, Kiaris *et al*. showed the importance of Ras and Notch in cyclin D1-dependent mammary oncogenesis by transgenic expression of the Notch antagonist Deltex (76). In this mouse mammary tumor model, H-Ras and Notch up-regulated expression of cyclin D1, suggesting that the mode of cooperation might be due to convergent up-regulation of a common target (72). Collectively, emerging evidence suggests that oncogenes such as *Ras*, *cyclin D1*, growth factors and growth factor receptors lead to the activation of Notch signaling whose cross-talk with other signaling pathways results in tumor development and progression.

Notch and Cancer Stem Cells

Recently, several reports have described molecular connections between Notch regulated transcription factors and pathways in controlling stem cell function, which further suggest that a new mechanism exists in support of the claim that Notch may drive tumor growth through the generation or expansion of tumor-initiating cells or cancer stem-like cells (CSCs) (4). Stem cells are defined by their capacity for self-renewal and differentiate into the full spectrum of cells characterizing a particular organism or tissue. Stem cells are of three major types embryonic, germinal and somatic (77). The inner cell mass of the

blastocyst generates embryonic stem cells. The embryonic stem cells are omnipotent, capable of generating any cell in the mature organism and have unlimited capacity to replicate. Germinal stem cells come from the germinal layer of the embryo. These germinal stem cells differentiate to generate specific organs. Somatic stem cells have the capacity to self-renew and differentiate into all cells characteristic of a specific organ or tissue (77). Stem cells often stay at locations that are called stem cell niches. Specifically, stem cell niches are defined as particular locations or microenvironments that allow the combined properties of stem cell self-renewal and multi-potency to be maintained (78). A combination of genetic and molecular analyses has identified many factors that support stem cell niches that also control stem cell identity. These factors include components of the Notch, Wnt and Shh signaling pathways (79).

Emerging evidence suggests that the capability of a tumor to grow and propagate is dependent on a small subset of cells within the tumor, termed CSCs. CSCs have been identified and isolated from tumors of the hematopoietic system, breast, lung, prostate, colon, brain, head and neck and pancreas (80-85). CSCs are able to self-renew, differentiate and regenerate phenotypic cells of the original tumor when implanted into severe combined immunodeficient mice (84, 85). These cells are identified by specific stem cell markers, antigens, molecules and signaling pathways (86).

The pathways that regulate self-renewal and cell fate in these systems are beginning to be elucidated. Transcription factors and molecules associated with oncogenesis, such as Notch, NF-κB, B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), Wnt, Shh and their biochemical pathways, are active only in a small minority of cancer cells where they may play key roles in determining the biological behavior of a tumor (86). Katoh reported that the balance between Wnt-fibroblast growth factor (FGF)-Notch and bone morphogenetic protein (BMP)-hedgehog signaling networks is important for the maintenance of homeostasis among stem and progenitor cells. Disruption of the stem cell signaling network results in pathological conditions, such as congenital diseases and cancer (87). In addition to pathways such as Wnt, Notch and hedgehog, known to regulate self-renewal of normal stem cells, tumor suppressor genes such as *PTEN* and *p53* have also been implicated in the regulation of cancer stem cell self-renewal (87).

Phillips *et al.* reported that cancer stem cells can be identified by phenotypic markers and their fate is controlled by the Notch pathway in breast cancer (88). Recombinant human erythropoietin receptor increased the numbers of stem cells and the self-renewing capacity in a Notch-dependent fashion by the induction of the Notch ligand, Jagged-1. Inhibitors of the Notch pathway blocked this effect,

suggesting the mechanistic role of Notch signaling in the maintenance of the CSC phenotype (88). Farnie *et al.* also provided evidence for breast cancer stem cells and their studies have consistently shown that stem-like cells and breast cancer-initiating populations can be enriched using the cell surface markers CD44+/CD24– that showed up-regulated genes including *Notch* (89). Notch signaling also promotes the formation of CSCs in human glioma. The overexpression of Notch-1 in SHG-44 glioma cells promoted their growth and colony-forming activity. Interestingly, the overexpression of ICN increased the formation of neurosphere-like colonies in the presence of growth factors. These colonies expressed nestin and also expressed neuron-, astrocyte- or oligodendrocyte-specific markers, consistent with phenotypes of neural stem cells. These data suggest potential functions of the Notch pathway in the formation of CSCs in human glioma (90). Recently, Fan *et al.* found that Notch blockade reduced the CD133-positive cell fraction almost 5-fold and totally abolished the side population, suggesting that the loss of tumor-forming capacity could be due to the depletion of CSCs. Notch signaling levels were higher in the CSC fraction, providing a potential mechanism for their increased sensitivity to the inhibition of this pathway. They also observed that apoptotic rates following Notch blockade were almost 10-fold higher in primitive nestin-positive cells as compared with nestin-negative cells. CSCs in brain tumors thus seem to be selectively vulnerable to agents inhibiting the Notch pathway (91). Moreover, Jagged-2, a Notch ligand, was found to be overexpressed in the leukemic stem cell (LSC) samples. *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT), an inhibitor of γ-secretase, a protease that is involved in Jagged and Notch signaling, inhibited LSC growth in colony formation assays (92). Taken together, these results suggested that the Notch pathway plays an important role not only in normal stem cells, but also in cancer stem cells.

Notch as a Cancer Treatment Target

A growing body of literature strongly suggests that increased expression of *Notch* genes and their ligands are detected in many human cancer cells and tissues such as those of the pancreas, breast, lung, mesothelioma, head and neck, renal, cervical, ovarian, endometrial, osteosarcoma, glioma and medulloblastoma, and leukemia (1, 2, 5, 93-95). These results clearly suggest that inactivation of Notch signaling by novel approaches would have a significant impact in cancer therapy. Moreover, current cancer therapeutics based on tumor regression may target and kill differentiated tumor cells, which make up the bulk of the tumor, while sparing the rare cancer stem cell population that must be killed for successful therapy. The cancer stem

cell model suggests that the design of new cancer therapeutics may require the targeting and elimination of cancer stem cells. Therefore eradicating cancer stem cells is increasingly being recognized as an important goal in curing cancer and thus the Notch pathway is considered an attractive target for treatment. Reducing Notch activity in cancer stem cells may promote their differentiation, thus reducing their ability to repopulate the cells forming the tumor mass. Recently, Notch was reported to regulate interleukin-10 (IL-10) production by T-helper 1 (Th1) cells (96) and suppress immunity. Since Th1 cells and their products are known to mediate antitumor responses, Notch-induced IL-10 production by Th1 cells can self-regulate Th1 cytokine production patterns leading to the suppression of Th1 cell-induced delayed–type hypersensitivity. IL-10 production can be elicited by all four mammalian Notch receptors (96), which also suggests that the inactivation of Notch could also revert immune suppression so that the cells could be killed *via* T-cell-mediated killing.

Moreover, dendritic cells (DCs) acquire the capacity for Dll-4 expression upon stimulation with Toll-like receptor (TLR) ligands and simultaneously induce IL-10 production by Th1 cells *in vitro* and *in vivo*. On the other hand, TLR ligation up-regulates the expression of Notch ligands Dll-1 or Dll-4 *via* the myeloid differentiation primary response gene 88 (MyD88) pathway which strongly inhibits Th2 cell development (97). Therefore, it is clear that the Notch pathway may play a pivotal role in the development of specific immune responses in Th1 and Th2 cells as well as DC activation and differentiation. The interruption of Notch signaling by deletion of Notch+ tumor cells may not only eliminate clonogenic tumor cells, but more importantly, serve to interrupt the tolerance of immunosuppressive factors or circuitry induced by tumor cells by the interruption of the production of IL-10 or other suppressive substances to reverse immune suppression.

Notch signaling is activated *via* the activity of γ-secretase which makes it a target in cancer therapy. Several forms of γ-secretase inhibitors have been tested for antitumor effects. For example, IL-X, an original γ-secretase inhibitor, has been shown to have Notch-1-dependent antitumor activity in Ras-transformed fibroblasts (75). Recently, the dipeptide γ-secretase inhibitor DAPT was reported to suppress medulloblastoma growth and induce G_0-G_1 cell cycle arrest and apoptosis in a T-ALL animal model (65, 98). Treatment with tripeptide γ-secretase inhibitor resulted in a marked reduction in tumor growth in cell lines and xenografts from melanoma and Kaposi sarcoma in mice (99). Dibenzazepine, one of the γ-secretase inhibitors, has been reported to inhibit epithelial cell proliferation and induce goblet cell differentiation in intestinal adenomas (68). We also found that a γ-secretase inhibitor suppressed prostate cancer cell growth (100).

Inhibitors of γ-secretase are being tested in Phase I clinical trials, suggesting that Notch signaling is an important target in cancer therapy. However, one of the major challenges is to eliminate unwanted toxicity associated with γ-secretase inhibitors, especially cytotoxicity in the gastrointestinal tract (101). Shih *et al*. reported the possible mechanisms underlying the unwanted cytotoxicity of γ-secretase inhibitors (93). Firstly, the Notch signaling pathway is known to widely participate in cellular physiology in normal tissues, including hematopoiesis and the maintenance of arterial smooth muscle, therefore, it is plausible that inactivation of γ-secretase may lead to the dysfunction of vital organs. Secondly, γ-secretase inhibitors do not exclusively target the Notch signaling pathways because γ-secretase has many substrates in addition to Notch receptors, such as several Notch ligands, v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ErbB4) and CD44. Thirdly, γ-secretase inhibitors may target proteases other than γ-secretase. Therefore, γ-secretase inhibitors may have widespread adverse effects *in vivo* because proteases participate in a wide array of cellular functions (93).

Studies from our laboratory have shown that chemopreventive agents such as genistein and curcumin (non-toxic agents from dietary sources) may inhibit Notch-1 activation in pancreatic cancer cells leading to apoptotic cell death (102, 103). A Chinese herb mixture (antitumor B) also inhibited Notch expression in a mouse lung tumor model (104). Recently, resveratrol has also been shown to induce apoptosis by inhibiting the Notch pathway mediated by p53 and PI3K/Akt in T-ALL (105). These findings suggest that Notch-1 down-regulation, especially by genistein or curcumin, could be a novel therapeutic approach for the treatment of human malignancies by targeting the inactivation of Notch signaling. However, further in-depth studies including mechanistic *in vitro* studies, *in vivo* animal experiments and clinical trials are needed to fully appreciate the consequence of the down-regulation of Notch-1 signaling by non-toxic dietary chemopreventive agents. We believe that this article could stimulate further research in this field for the development of non-toxic approaches for cancer therapy by targeting Notch signaling, which is likely to eliminate not only tumor cells, but also cancer stem cells, in addition to reverting immune suppression.

Acknowledgements

References

1 Miele L: Notch signaling. Clin Cancer Res *12*: 1074-1079, 2006.

2 Miele L, Miao H and Nickoloff BJ: NOTCH signaling as a novel cancer therapeutic target. Curr Cancer Drug Targets *6*: 313-323, 2006.

3 Okuyama R, Tagami H and Aiba S: Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci *49*: 187-194, 2008.

4 Wilson A and Radtke F: Multiple functions of Notch signaling in self-renewing organs and cancer. FEBS Lett *580*: 2860-2868, 2006.

5 Miele L and Osborne B: Arbiter of differentiation and death: Notch signaling meets apoptosis. J Cell Physiol *181*: 393-409, 1999.

6 Joutel A and Tournier-Lasserve E: Notch signalling pathway and human diseases. Semin Cell Dev Biol *9*: 619-625, 1998.

7 Qi R, An H, Yu Y *et al*: Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer Res *63*: 8323-8329, 2003.

8 Shou J, Ross S, Koeppen H, de Sauvage FJ and Gao WQ: Dynamics of notch expression during murine prostate development and tumorigenesis. Cancer Res *61*: 7291-7297, 2001.

9 Sriuranpong V, Borges MW, Ravi RK *et al*: Notch signaling induces cell cycle arrest in small cell lung cancer cells. Cancer Research *61*: 3200-3205, 2001.

10 Miyamoto Y, Maitra A, Ghosh B *et al*: Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell *3*: 565-576, 2003.

11 Koch U and Radtke F: Notch and cancer: a double-edged sword. Cell Mol Life Sci *64*: 2746-2762, 2007.

12 Allenspach EJ, Maillard I, Aster JC and Pear WS: Notch signaling in cancer. Cancer Biol Ther *1*: 466-476, 2002.

13 Bolos V, Grego-Bessa J and de la Pompa JL: Notch signaling in development and cancer. Endocr Rev *28*: 339-363, 2007.

14 Chiaramonte R, Basile A, Tassi E *et al*: A wide role for NOTCH1 signaling in acute leukemia. Cancer Lett *219*: 113-120, 2005.

15 Aster JC, Pear WS and Blacklow SC: Notch signaling in leukemia. Annu Rev Pathol *3*: 587-613, 2008.

16 Reedijk M, Odorcic S, Chang L *et al*: High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Research *65*: 8530-8537, 2005.

17 Zhu YM, Zhao WL, Fu JF *et al*: *NOTCH1* mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. Clin Cancer Res *12*: 3043-3049, 2006.

18 Santagata S, Demichelis F, Riva A *et al*: JAGGED1 expression is associated with prostate cancer metastasis and recurrence. Cancer Res *64*: 6854-6857, 2004.

19 Aggarwal BB: Nuclear factor-kappaB: the enemy within. Cancer Cell *6*: 203-208, 2004.

20 Sarkar FH and Li Y: NF-kappaB: a potential target for cancer chemoprevention and therapy. Front Biosci *13*: 2950-2959, 2008.

21 Oswald F, Liptay S, Adler G and Schmid RM: *NF-kappaB2* is a putative target gene of activated Notch-1 *via* RBP-Jkappa. Mol Cell Biol *18*: 2077-2088, 1998.

22 Jang MS, Miao H, Carlesso N *et al*: Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. J Cell Physiol *199*: 418-433, 2004.

23 Nickoloff BJ, Qin JZ, Chaturvedi V, Denning MF, Bonish B and Miele L: Jagged-1-mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. Cell Death Differ *9*: 842-855, 2002.

24 Wang Y, Chan SL, Miele L *et al*: Involvement of Notch signaling in hippocampal synaptic plasticity. Proc Natl Acad Sci USA *101*: 9458-9462, 2004.

25 Vilimas T, Mascarenhas J, Palomero T *et al*: Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. Nat Med *13*: 70-77, 2007.

26 Fernandez-Majada V, Aguilera C, Villanueva A *et al*: Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. Proc Natl Acad Sci USA *104*: 276-281, 2007.

27 Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y and Sarkar FH: Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{kappa}B, vascular endothelial growth factor and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res *66*: 2778-2784, 2006.

28 Bash J, Zong WX, Banga S *et al*: Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. EMBO J *18*: 2803-2811, 1999.

29 Moran ST, Cariappa A, Liu H *et al*: Synergism between NF-kappa B1/p50 and Notch2 during the development of marginal zone B lymphocytes. J Immunol *179*: 195-200, 2007.

30 Osipo C, Golde TE, Osborne BA and Miele LA: Off the beaten pathway: the complex cross talk between Notch and NF-kappaB. Lab Invest *88*: 11-17, 2008.

31 Crowell JA, Steele VE and Fay JR: Targeting the AKT protein kinase for cancer chemoprevention. Mol Cancer Ther *6*: 2139-2148, 2007.

32 Cheng GZ, Park S, Shu S *et al*: Advances of AKT pathway in human oncogenesis and as a target for anti-cancer drug discovery. Curr Cancer Drug Targets *8*: 2-6, 2008.

33 Tokunaga E, Oki E, Egashira A *et al*: Deregulation of the Akt pathway in human cancer. Curr Cancer Drug Targets *8*: 27-36, 2008.

34 Brunet A, Bonni A, Zigmond MJ *et al*: Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*: 857-868, 1999.

35 Cardone MH, Roy N, Stennicke HR *et al*: Regulation of cell death protease caspase-9 by phosphorylation. Science *282*: 1318-1321, 1998.

36 Rommel C, Clarke BA, Zimmermann S *et al*: Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science *286*: 1738-1741, 1999.

37 Kane LP, Mollenauer MN, Xu Z, Turck CW and Weiss A: Akt-dependent phosphorylation specifically regulates Cot induction of *NF-kappa B*-dependent transcription. Mol Cell Biol *22*: 5962-5974, 2002.

38 Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM and Donner DB: NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature *401*: 82-85, 1999.

39 Liu ZJ, Xiao M, Balint K *et al*: Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. Cancer Res *66*: 4182-4190, 2006.

40 Palomero T, Dominguez M and Ferrando AA: The role of the PTEN/AKT pathway in NOTCH1-induced leukemia. Cell Cycle *7*: 965-970, 2008.

41 Gutierrez A and Look AT: NOTCH and PI3K-AKT pathways intertwined. Cancer Cell *12*: 411-413, 2007.

42 Palomero T, Sulis ML, Cortina M *et al*: Mutational loss of *PTEN* induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med *13*: 1203-1210, 2007.

43 Veeraraghavalu K, Subbaiah VK, Srivastava S, Chakrabarti O, Syal R and Krishna S: Complementation of human papillomavirus type 16 E6 and E7 by Jagged1-specific Notch1-phosphatidylinositol 3-kinase signaling involves pleiotropic oncogenic functions independent of CBF1;Su(H);Lag-1 activation. J Virol *79*: 7889-7898, 2005.

44 Androutsellis-Theotokis A, Leker RR, Soldner F *et al*: Notch signalling regulates stem cell numbers *in vitro* and *in vivo*. Nature *442*: 823-826, 2006.

45 Chan SM, Weng AP, Tibshirani R, Aster JC and Utz PJ: Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. Blood *110*: 278-286, 2007.

46 Mungamuri SK, Yang X, Thor AD and Somasundaram K: Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Research *66*: 4715-4724, 2006.

47 Fingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C and Blenis J: mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Mol Cell Biol *24*: 200-216, 2004.

48 Chiang GG and Abraham RT: Targeting the mTOR signaling network in cancer. Trends Mol Med *13*: 433-442, 2007.

49 Purow BW, Sundaresan TK, Burdick MJ *et al*: Notch-1 regulates transcription of the epidermal growth factor receptor through p53. Carcinogenesis *29*: 918-925, 2008.

50 Zhang YW, Wang R, Liu Q, Zhang H, Liao FF and Xu H: Presenilin/gamma-secretase-dependent processing of beta-amyloid precursor protein regulates EGF receptor expression. Proc Natl Acad Sci USA *104*: 10613-10618, 2007.

51 Wang Z, Sengupta R, Banerjee S *et al*: Epidermal growth factor receptor-related protein inhibits cell growth and invasion in pancreatic cancer. Cancer Research *66*: 7653-7660, 2006.

52 Kong D, Banerjee S, Huang W *et al*: Mammalian target of rapamycin repression by 3,3'-diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells. Cancer Res *68*: 1927-1934, 2008.

53 Wang Z, Kong D, Banerjee S *et al*: Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling. Cancer Res *67*: 11377-11385, 2007.

54 Fredriksson L, Li H and Eriksson U: The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev *15*: 197-204, 2004.

55 Reigstad LJ, Varhaug JE and Lillehaug JR: Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. FEBS J *272*: 5723-5741, 2005.

56 Li H, Fredriksson L, Li X and Eriksson U: PDGF-D is a potent transforming and angiogenic growth factor. Oncogene *22*: 1501-1510, 2003.

57 Bongarzone ER, Byravan S, Givogri MI, Schonmann V and Campagnoni AT: Platelet-derived growth factor and basic fibroblast growth factor regulate cell proliferation and the expression of notch-1 receptor in a new oligodendrocyte cell line. J Neurosci Res *62*: 319-328, 2000.

58 Claxton S and Fruttiger M: Periodic Delta-like 4 expression in developing retinal arteries. Gene Expr Patterns *5*: 123-127, 2004.

59 Ingram WJ, McCue KI, Tran TH, Hallahan AR and Wainwright BJ: Sonic hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling. Oncogene *27*: 1489-1500, 2008.

60 Pepinsky RB, Rayhorn P, Day ES *et al*: Mapping sonic hedgehog-receptor interactions by steric interference. J Biol Chem *275*: 10995-11001, 2000.

61 Nicolas M, Wolfer A, Raj K *et al*: Notch1 functions as a tumor suppressor in mouse skin. Nat Genet *33*: 416-421, 2003.

62 Katoh M: Dysregulation of stem cell signaling network due to germline mutation, SNP, *helicobacter pylori* infection, epigenetic change and genetic alteration in gastric cancer. Cancer Biol Ther *6*: 832-839, 2007.

63 Yokota N, Mainprize TG, Taylor MD *et al*: Identification of differentially expressed and developmentally regulated genes in medulloblastoma using suppression subtraction hybridization. Oncogene *23*: 3444-3453, 2004.

64 Dakubo GD, Mazerolle CJ and Wallace VA: Expression of Notch and Wnt pathway components and activation of Notch signaling in medulloblastomas from heterozygous patched mice. J Neurooncol *79*: 221-227, 2006.

65 Hallahan AR, Pritchard JI, Hansen S *et al*: The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. Cancer Res *64*: 7794-7800, 2004.

66 Nakamura T, Tsuchiya K and Watanabe M: Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. J Gastroenterol *42*: 705-710, 2007.

67 Proweller A, Tu L, Lepore JJ *et al*: Impaired notch signaling promotes *de novo* squamous cell carcinoma formation. Cancer Research *66*: 7438-7444, 2006.

68 van Es JH, van Gijn ME, Riccio O *et al*: Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature *435*: 959-963, 2005.

69 Duncan AW, Rattis FM, DiMascio LN *et al*: Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol *6*: 314-322, 2005.

70 Trowbridge JJ, Xenocostas A, Moon RT and Bhatia M: Glycogen synthase kinase-3 is an *in vivo* regulator of hematopoietic stem cell repopulation. Nat Med *12*: 89-98, 2006.

71 Blank U, Karlsson G and Karlsson S: Signaling pathways governing stem-cell fate. Blood 111: 492-503, 2008.

72 Sundaram MV: The love-hate relationship between Ras and Notch. Genes Dev 19: 1825-1839, 2005.

73 Stockhausen MT, Sjolund J and Axelson H: Regulation of the Notch target gene *Hes-1* by TGFalpha-induced Ras/MAPK signaling in human neuroblastoma cells. Exp Cell Res *310*: 218-228, 2005.

74 Fitzgerald K, Harrington A and Leder P: Ras pathway signals are required for notch-mediated oncogenesis. Oncogene 19: 4191-4198, 2000.

75 Weijzen S, Rizzo P, Braid M et al: Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 8: 979-986, 2002.

76 Kiaris H, Politi K, Grimm LM et al: Modulation of notch signaling elicits signature tumors and inhibits hras1-induced oncogenesis in the mouse mammary epithelium. Am J Pathol 165: 695-705, 2004.

77 Kakarala M and Wicha MS: Cancer stem cells: implications for cancer treatment and prevention. Cancer J 13: 271-275, 2007.

78 Ohlstein B, Kai T, Decotto E and Spradling A: The stem cell niche: theme and variations. Curr Opin Cell Biol 16: 693-699, 2004.

79 Keith B and Simon MC: Hypoxia-inducible factors, stem cells and cancer. Cell 129: 465-472, 2007.

80 Rossi DJ, Jamieson CH and Weissman IL: Stems cells and the pathways to aging and cancer. Cell 132: 681-696, 2008.

81 Cariati M and Purushotham AD: Stem cells and breast cancer. Histopathology 52: 99-107, 2008.

82 Sakariassen PO, Immervoll H and Chekenya M: Cancer stem cells as mediators of treatment resistance in brain tumors: status and controversies. Neoplasia 9: 882-892, 2007.

83 Lagasse E: Cancer stem cells with genetic instability: the best vehicle with the best engine for cancer. Gene Ther 15: 136-142, 2008.

84 Tang C, Ang BT and Pervaiz S: Cancer stem cell: target for anticancer therapy. FASEB J 21: 3777-3785, 2007.

85 Sales KM, Winslet MC and Seifalian AM: Stem cells and cancer: an overview. Stem Cell Rev 3: 249-255, 2007.

86 Styczynski J and Drewa T: Leukemic stem cells: from metabolic pathways and signaling to a new concept of drug resistance targeting. Acta Biochim Pol 54: 717-726, 2007.

87 Katoh M: Networking of WNT, FGF, Notch, BMP and Hedgehog signaling pathways during carcinogenesis. Stem Cell Rev 3: 30-38, 2007.

88 Phillips TM, Kim K, Vlashi E, McBride WH and Pajonk F: Effects of recombinant erythropoietin on breast cancer-initiating cells. Neoplasia 9: 1122-1129, 2007.

89 Farnie G and Clarke RB: Mammary stem cells and breast cancer – role of Notch signalling. Stem Cell Rev 3: 169-175, 2007.

90 Zhang XP, Zheng G, Zou L et al: Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. Mol Cell Biochem 307: 101-108, 2008.

91 Fan X, Matsui W, Khaki L et al: Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. Cancer Research 66: 7445-7452, 2006.

92 Gal H, Amariglio N, Trakhtenbrot L et al: Gene expression profiles of AML-derived stem cells; similarity to hematopoietic stem cells. Leukemia 20: 2147-2154, 2006.

93 Shih I and Wang TL: Notch signaling, gamma-secretase inhibitors and cancer therapy. Cancer Res 67: 1879-1882, 2007.

94 Sjolund J, Manetopoulos C, Stockhausen MT and Axelson H: The Notch pathway in cancer: differentiation gone awry. Eur J Cancer 41: 2620-2629, 2005.

95 Wang Z, Zhang Y, Li Y, Banerjee S, Liao J and Sarkar FH: Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. Mol Cancer Ther 5: 483-493, 2006.

96 Rutz S, Janke M, Kassner N, Hohnstein T, Krueger M and Scheffold A: Notch regulates IL-10 production by T-helper 1 cells. Proc Natl Acad Sci USA 105: 3497-3502, 2008.

97 Sun J, Krawczyk CJ and Pearce EJ: Suppression of Th2 cell development by Notch ligands Delta1 and Delta4. J Immunol 180: 1655-1661, 2008.

98 O'Neil J, Calvo J, McKenna K et al: Activating Notch1 mutations in mouse models of T-ALL. Blood 107: 781-785, 2006.

99 Curry CL, Reed LL, Golde TE, Miele L, Nickoloff BJ and Foreman KE: Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. Oncogene 24: 6333-6344, 2005.

100 Zhang Y, Wang Z, Ahmed F, Banerjee S, Li Y and Sarkar FH: Down-regulation of Jagged-1 induces cell growth inhibition and S-phase arrest in prostate cancer cells. Int J Cancer 119: 2071-2077, 2006.

101 Barten DM, Meredith JE Jr, Zaczek R, Houston JG and Albright CF: Gamma-secretase inhibitors for Alzheimer's disease: balancing efficacy and toxicity. Drugs R D 7: 87-97, 2006.

102 Wang Z, Zhang Y, Banerjee S, Li Y and Sarkar FH: Inhibition of nuclear factor kappaB activity by genistein is mediated via Notch-1 signaling pathway in pancreatic cancer cells. Int J Cancer 118: 1930-1936, 2006.

103 Wang Z, Zhang Y, Banerjee S, Li Y and Sarkar FH: Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. Cancer 106: 2503-2513, 2006.

104 Zhang Z, Wang Y, Yao R et al: Cancer chemopreventive activity of a mixture of Chinese herbs (antitumor B) in mouse lung tumor models. Oncogene 23: 3841-3850, 2004.

105 Cecchinato V, Chiaramonte R, Nizzardo M et al: Resveratrol-induced apoptosis in human T-cell acute lymphoblastic leukaemia MOLT-4 cells. Biochem Pharmacol 74: 1568-1574, 2007.

Review

Emerging roles of PDGF-D signaling pathway in tumor development and progression

Zhiwei Wang *, Aamir Ahmad, Yiwei Li, Dejuan Kong, Asfar S. Azmi, Sanjeev Banerjee, Fazlul H. Sarkar *

Department of Pathology, Karmanos Cancer Institute, Wayne State University, School of Medicine, Detroit, MI 48201, USA

A R T I C L E   I N F O

A B S T R A C T

Platelet-derived growth factor-D (PDGF-D) can regulate many cellular processes, including cell proliferation, apoptosis, transformation, migration, invasion, angiogenesis and metastasis. Therefore PDGF-D signaling has been considered to be important in human malignancies, and thus PDGF-D signaling may represent a novel therapeutic target, and as such suggests that the development of agents that will target PDGF-D signaling is likely to have a significant therapeutic impact on human cancers. This mini-review describes the mechanisms of signal transduction associated with PDGF-D signaling to support the role of PDGF-D in the carcinogenesis. Moreover, we summarize data on several PDGF-D inhibitors especially naturally occurring "chemopreventive agent" such an indole compound, which we believe could serve as a novel agent for the prevention of tumor progression and/or treatment of human malignancies by targeted inactivation of PDGF-D signaling.

© 2010 Elsevier B.V. All rights reserved.

Contents

---

1. Introduction

Platelet-derived growth factor (PDGF) signaling pathway has been extensively studied and well characterized since PDGF was first described in the 1970s as a serum factor that promoted the smooth muscle cell proliferation [1]. The PDGF family is comprised of four different polypeptide chains encoded by different genes, which have been identified: PDGF-A, PDGF-B, and recently discovered PDGF-C and PDGF-D [2–4]. PDGF need to be assembled into disulphide-bonded dimmers via homodimerization or heterodimerization in order to play their functional role. So far, four homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD, and one heterodimer, PDGF-AB have been described [5]. It is interesting to note that no heterodimers involving PDGF-C and PDGF-D chains have been described. In addition, it is notable that PDGF-A and PDGF-B are secreted in their active forms, while PDGF-C and PDGF-D are secreted as inactive forms requiring activation for their function. These differences could be due to their subtle structural differences although the PDGF have a common structure with typical growth factor domain involved in the dimerization of the two subunits, and in terms of their receptor binding and activation. Interestingly, the PDGF show high sequence identity with the vascular endothelial growth factor (VEGF), which may suggest their similar but distinct biological functions. Therefore, PDGF family is sometimes referred to as PDGF/VEGF family [1]. PDGF-A and PDGF-B mainly encode the growth factor domain and have short N-terminal extensions that undergo intracellular proteolytic processing for activation, while both PDGF-C and PDGF-D chains encode a unique two-domain structure with an N-terminal 'Clr/Cls, urchin endothelial growth factor-like (UEGF) protein and bone morphogenic protein 1' (CUB) domain, as part of their N-terminal extensions, in addition to the C-terminal growth factor domain. The basic domain structure of PDGF family members is provided in Fig. 1.

Several reports have shown that the CUB domains of PDGF-D have to be cleaved extracellularly to make the COOH-terminal growth factor domain active for PDGF-D binding to its receptor. PDGFs exert their cellular effects by activating two structurally related receptor tyrosine kinases of the PDGF (PDGFR), PDGFR-α and PDGFR-β. The PDGF-AA activates PDGFRα, whereas PDGF-BB activates PDGFR-α, PDGFR-α/β and PDGFR-β. PDGF-AB and PDGF-CC activate PDGFR-α and PDGFR-α/β, while PDGF-DD specifically binds to and activates its

cognate receptor PDGFR-β (Fig. 1). The physiological relevance of the ability of PDGFs to activate PDGFR heterodimers is unclear at present [1]. The phosphorylation of PDGF receptor triggers a number of downstream signaling pathways including activation of phosphatidylinositol 3 kinase (PI3K), Akt, nuclear factor-κB (NF-κB), Notch, extracellular signal-regulated kinase (ERK), etc. [5–8]. Although all four PDGF ligands play their oncogenic roles through two PDGFRs, they could promote carcinogenesis through different targets. Without doubt further investigations are needed to elucidate how PDGF ligands active different PDGFR downstream genes.

2. PDGF-D, the latest member of PDGF family, has unique functions

As mentioned above, all the PDGF isoforms elicits their biological functions through a total of two receptors. While this leads to overlapping cellular effects in some cases, it is increasingly being realized that different PDGFs exhibit mutually exclusive physiological effects as well [9]. Thus, PDGF signaling is a complex pathway, more so because of the addition of latest members, PDGF-C and PDGF-D [10]. Recognition of these new factors has, in spite of adding complexity to overall understanding of PDGF signaling, helped explain some key developmental processes, which shed more light on the overall interplay and regulation of these growth factors. For example, in transdifferentiating hepatic stellate cells, different PDGF isoforms have been shown to be expressed at different stages [11]. PDGF-B is expressed predominantly at initial stages, whereas PDGF-D takes over at transitional stage while the levels of PDGF-C are high at later stages. This indicates that while different PDGFs might contribute to the same physiological process, each PDGF has a different and distinct role.

The report by Lokker et al. is a good example of how perception of PDGF signaling has changed with the discovery of PDGF-D [12]. This study was focused on glioblastoma multiforme, a particularly aggressive brain tumor, where the median survival time is just 9–12 months. Before this study, PDGF-B was believed to be a crucial factor involved in growth of such brain tumors primarily because it was the only PDGF family member known to function through PDGFR-β [13]. This notion, however, changed with the detailed characterization of PDGF-D, along with other PDGFs, and the two receptors, studied in 11 glioma cell lines [12]. PDGF-D was found to be expressed in 10 of the 11 cell lines and PDGFR-β in 9 of 11 cell lines whereas
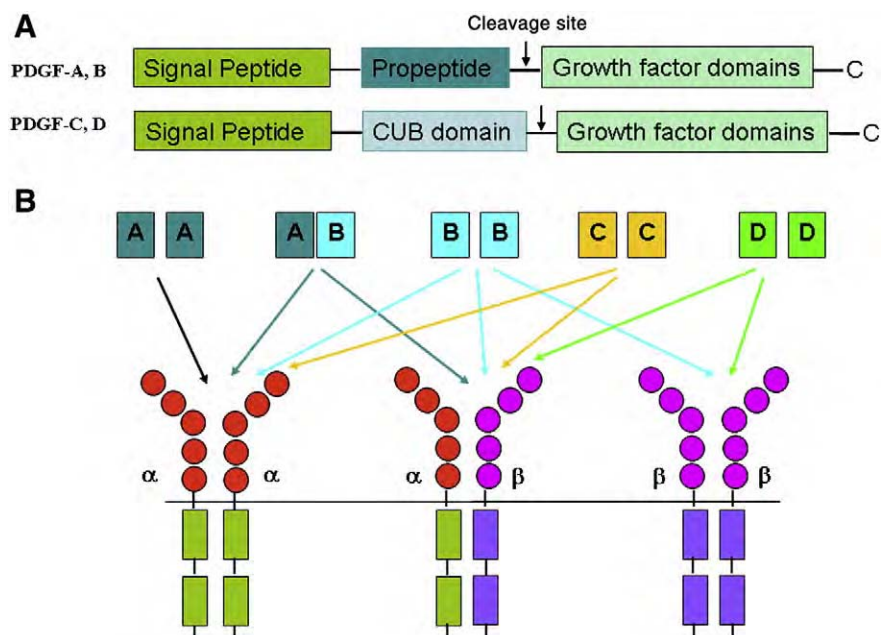

Fig. 1. A; Schematic drawing of the four PDGF proteins (PDGF-A, B, C and D). B; Demonstrates the PDGF–PDGFR interactions.

PDGF-B was expressed in only 5 of the 11 cell lines. It was thus realized that PDGF-D and PDGFR-β might be the player of a novel autocrine loop that does not involve PDGF-B. These results suggest that PDGF-D can activate PDGFR-β and influence the aggressiveness of tumors without the involvement of classical PDGFR-β ligand PDGF-B.

In an earlier study [14], PDGF-D mRNA transcript was detected in many ovarian, lung, renal and brain cancer-derived cell lines. Further, a direct comparison between PDGF-B, PDGF-D and PDGFR-β in central nervous system-derived cancer cell lines in this study revealed that PDGF-D is expressed in 10 of 17 cell lines, PDGF-B in only 7 and PDGFR-β can be detected in 9 of those 17 cell lines. A total of five cell lines expressed only PDGF-D while there were only two cell lines that expressed only PDGF-B. Interestingly, PDGFR-β was detected in all of these 7 cell lines. These observations seem to suggest that a) PDGF-D might be a better molecular marker for aggressiveness than PDGF-B, and b) PDGF-D signaling can function independent of PDGF-B even though both activate PDGFR-β. Clearly, more investigations are needed to spell out the differences between PDGF-B and PDGF-D signaling pathways.

Based on these few preliminary reports, a consensus seem to emerge which suggest that PDGF-D might play a definitive role in human cancers through independent regulation of cellular signaling pathways. The other PDGF family members, particularly PDGF-A and PDGF-B, have been studied and reviewed in considerable detail [15–24] and because of that reason, we specifically focused our discussion on PDGF-D, the latest member of this family, for thus review article. Therefore, in the subsequent paragraphs we are presenting the biological functions of PDGF-D in human malignancies.

3. The role of PDGF-D in cancer

Since PDGF-A and PDGF-B has been well documented and characterized; however their roles in human cancers are questionable, thus we will not discuss their roles in cancer progression in this article. In spite of the discovery of PDGF-D over 10 years ago, the role of PDGF-D is just beginning to be understood. The growing body of literature strongly suggests that PDGF-D may function as a key player in the development and progression of human cancers by regulating the processes of cell proliferation, apoptosis, migration, invasion, angiogenesis, and metastasis. It has been reported that PDGF-D signaling is frequently deregulated in human malignancies with up-regulated expression of PDGF-D was found in prostate, lung, renal, ovarian, brain, and pancreatic cancer [7,8,12,25–27]. Ustach et al. found that human prostate carcinoma LNCaP cells are capable of processing full-length PDGF-D into a biologically active PDGF form which binds and activate its cognate PDGFR-β. Moreover, PDGF-D expression greatly accelerates the tumor growth and enhances prostate carcinoma cell interaction with the surrounding stromal layers in a severe combined immunodeficient (SCID) mouse model, suggesting the potential oncogenic activity of PDGF-D in human prostate cancer progression [25]. Pancreatic cancer like many other tumors has been shown to over-express the PDGF-D. We found that PDGF-D is highly expressed in human pancreatic adenocarcinoma specimens, in chronic pancreatitis associated with pancreatic adeno-carcinoma, and in different human pancreatic cancer cell lines, suggesting that PDGF-D could be important in human pancreatic cancer progression [8]. Moreover, Xu et al. reported that PDGF-D over-production in renal cancer SN12-C cells increased the proliferation and migration of cells *in vitro* and improved perivascular cell coverage *in vivo* [27]. Furthermore, blocking PDGF-D/PDGFR signaling inhibited survival and mitogenic pathways in the glioblastoma cell lines and prevented glioma formation in a nude mouse xenograft model [12].

The molecular mechanism(s) by which PDGF-D signaling induces tumor growth has not been fully elucidated. However, multiple oncogenic pathways, such as urokinase-type plasminogen activator (uPA), reactive oxygen species (ROS), proinflammatory cytokine interleukin-1β (IL-1β), phosphatidylinositol 3-kinase (PI3K)/Akt, NF-κB, Notch, ERK, mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), Cyclin D1, and Bcl-2 signaling have been reported to crosstalk with PDGF-D pathway, and thus it is believed that the crosstalk between PDGF-D and other signaling pathways plays important roles in tumor aggressiveness. Here, we will discuss the recent advances in our understanding on the role of PDGF-D in tumor progression. In this review, we will summarize the results of emerging studies on the PDGF-D signaling pathway, including the upstream regulators and the downstream effectors of the PDGF-D pathway, as well as its implication in human cancers. In addition, we sincerely apologize to those authors whose work could not be cited in this article because of space limitation.

4. Upstream regulators of PDGF-D

In recent years, studies on PDGF-D and cancer have burst onto the scene; however, the upstream regulators of PDGF-D in human cancer progression are largely unknown. The promoters for PDGF-A, PDGF-B as well as PDGF-C have been studied and our understanding of the mechanism of gene regulation of these three PDGFs is much more robust than that of PDGF-D [9]. Khachigian et al. have reported gene promoter studies for all the PDGFs. Their studies with PDGF-A and PDGF-C revealed the involvement of transcription factors Egr1 and Sp1 [28,29]. Egr1 was also found to interact with a novel element in PDGF-B promoter [30]. The investigations of this research group on the latest PDGF member, PDGF-D have identified Ets-1 and Sp1 as the transcription factors that regulates the expression of PDGF-D [31–33]. Clearly there has been a renewed interest to fully understand the gene regulation of PDGF-D. Also, available data points to differential regulation of PDGF-D gene compared to the other PDGF isoforms, thus pointing to the unique identity of PDGF-D among this family of growth factors.

In addition to transcription factors Ets-1 and Sp1, other factors such as uPA, H_2O_2, and IL-1β have also been reported to regulate the expression of PDGF-D signaling through different mechanisms [26,31,33]. H_2O_2 activates PDGF-D transcription and translation, whereas uPA is capable of processing recombinant latent PDGF-D into the active form through removal of the CUB domain. IL-1β suppresses PDGF-D promoter activity and mRNA and protein expression. The mechanisms by which these three upstream genes regulate PDGF-D are discussed in the following paragraphs.

4.1. Urokinase plasminogen activator (uPA) and its role in PDGF-D signaling

The urokinase plasminogen activator (uPA) system is a serine protease family comprising of urokinase-type plasminogen activator (uPA), plasminogen activator inhibitors (PAI's), tissue-type plasmin-ogen activator (tPA) and the uPA receptor (uPAR) [34]. It is well known that urokinase plasminogen system plays important roles in cell migration, angiogenesis, invasion and metastasis, and thus the spread of primary tumors to distant organs is in part associated uPA system, which correlate with poor prognosis, resulting in high mortality [35]. It has been reported that PDGF-D is activated by uPA [26]. We have indicated earlier that PDGF-DD is secreted as full-length, latent dimers, and the proteolytic cleavage of the CUB domain is required for the COOH-terminal growth factor domain to activate the PDGF receptor. Ustach et al. found that uPA is capable of processing recombinant latent PDGF-D into the active form through removal of the CUB domain. The uPA cleavage site resides at the R247/R249 within the hinge region between the CUB and the growth factor domains. Interestingly, closely related protease tPA did not activate the PDGF-D [26]. Prostate cancer cells PC-3 and LNCaP can auto-activate latent full-length PDGF-D into its biologically active form under serum-independent conditions. However, this auto-activation is inhibited by PAI-1, an uPA/tPA inhibitor, and the serine protease

inhibitor aprotinin. Very interestingly, uPA activates PDGF-D, which in turn regulates uPA expression and activity [26]. This evidence suggests that there is a direct link between uPA and PDGF-D-mediated cell signaling; however, the molecular mechanism of this feedback signaling loop is still unclear.

4.2. The role of reactive oxygen species (ROS) in PDGF-D signaling

Reactive oxygen species (ROS), continuously generated from mitochondrial respiratory chain, includes hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical ($\cdot OH$) and singlet oxygen [36]. In eukaryotic cells, the most significant intracellular sources of ROS are the mitochondrial respiratory chain, microsomal cytochrome P450 enzymes, flavoprotein oxidases, and peroxisomal fatty acid metabolism. Mammalian cells possesses an efficient antioxidant defense system, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione S-transferase (GST), catalase and peroxidases, and glutathione (GSH), which can scavenge the excessive ROS produced through cellular metabolism, and thus these antioxidant mechanisms when functioning correctly will make ROS level relatively stable under physiological conditions in order to maintain cellular homeostasis [37]. However, inefficient antioxidant mechanisms and/or over-production of ROS will lead to chronic diseases. It has been documented that when the intracellular homeostatic mechanism fails then the overproduced ROS could cause cellular oxidative stress, where DNA, lipids, proteins and other cellular components are oxidatively damaged. DNA damage induced by ROS is sufficient to convert normal cells to malignant cells [36]; therefore, aberrant ROS signaling has profound consequence in inducing human malignancies.

It has been reported that ROS is a signaling stimuli that is critical in mediating several cellular functions including tissue homeostasis and adaptation. A specific role of ROS in PDGF signaling has been suggested documenting that increased levels of ROS could lead to tyrosine phosphorylation of many proteins including the PDGF receptors [38,39]. PDGFR-β has been suggested to influence proliferation and migration [40] and chemotaxis [41] through an ROS-dependent pathway. Among the PDGF family, the involvement of ROS has been shown to influence PDGF-A, PDGF-B, and PDGF-D signaling pathways [31]. Liu et al. found that basal and inducible PDGF-D transcription is regulated by the winged helix-turn-helix factor and prototypic Ets family member, Ets-1 present in the PDGF-D promoter. Moreover, they found that H_2O_2 activates PDGF-D transcription at nanomolar concentration, and H_2O_2 also stimulates Ets-1 expression thereby linking ROS, regulation of Ets-1 and the regulation of PDGF-D transcription. Moreover, these authors have also demonstrated that angiotensin II, a well known factor that stimulate the production of ROS from NAPDH oxidase, could induce Ets-1 and PDGF-D expression via the endogenous generation of H_2O_2 [31], suggesting the importance of ROS in the regulation of Ets-1 and PDGF-D. Emerging evidence suggests that activation of PDGF receptors can be accomplished by factors other than PDGFs as well [1] and ROS seem to be crucial for this phenomenon [39]. Further research toward exploration of the molecular mechanisms by which ROS regulates PDGF-D requires in-depth investigations.

4.3. The role of interleukin-1β in the regulation of PDGF-D signaling

A growing body of evidence has shown that numerous cytokine polymorphisms are associated with increased risk of inflammatory diseases and cancer. Key proinflammatory cytokines include IL-1, -6, -8, -12 and -18, TNF-α and macrophage MIF (migration inhibitory factor). Anti-inflammatory cytokines include IL-4 and -10, IFN (interferon)-α and -β [42]. These cytokines have been linked to many immune reactions, including the recruitment of inflammatory cells to the site of infection. Moreover, the proinflammatory cytokines

are known to activate critical transcription factors such as NF-κB, AP-1, STAT1 and STAT3, all of which have been implicated in inflammation mediated development of tumors depending on cell types [42]. In pancreatic cancer, autocrine production of IL-1β has been reported to promote growth and conferring chemo-resistance to conventional therapeutic agents. Recently, the crosstalk between IL-1β and PDGF-D has been demonstrated. Li et al. reported that IL-1β could abolish the cell migration and proliferation induced by PDGF-D [43]. Moreover, Liu et al. reported that IL-1β suppresses PDGF-D promoter activity and mRNA and protein expression in a time-and dose-dependent manner [33]. IL-1β induced NF-κB p65 and interferon regulatory factor-1 (IRF-1) have been reported to bind to different elements in the PDGF-D promoter, leading to the inhibition of PDGF-D transcription. Furthermore, PDGF-D repression by IL-1β was reported to be mediated via histone deacetylation and interaction of histone deacetylase (HDAC)-1 with IRF-1 and p65 [33], suggesting that IL-1β inhibition of PDGF-D expression through IRF-1/p65/HDAC-1 may represent a negative regulatory mechanism, which could be a novel target for the inactivation of PDGF-D signaling.

5. Downstream effectors of PDGF-D signaling

PDGF-D regulates many cellular processes, including cell proliferation, transformation, invasion, and angiogenesis by specifically binding to and activating its cognate receptor PDGFR-β. Specifically, PDGF-D interacts with PDGFR-β and activates multiple downstream oncogenic pathways, resulting in tumor development and progression. For example, PDGF-D promotes cancer cell survival and growth through PI3K/Akt, mTOR, NF-κB, ERK, MAPK, and Notch pathway. PDGF-D increases cancer cell invasion, angiogenesis, and metastasis via up-regulation of VEGF, MMP, miRNA, E-cadherin, and snail expression. Here, we discuss the recent advances in the understanding on the role of PDGF-D in tumor progression.

5.1. Regulation of cell survival and growth

5.1.1. Phosphatidylinositol 3-kinase (PI3K)/Akt signaling and its crosstalks with PDGF-D signaling

PDGF-D has been reported to crosstalk with PI3K/Akt, which is an evolutionarily conserved serine/threonine kinase [7,44]. It is well accepted that crosstalk means that one signal will affect another neighboring signal pathway. Akt (also known as protein kinase B) is one of the major cell growth and apoptosis regulatory pathways. There are three isoforms of Akt such as Akt 1, Akt 2 and Akt 3, which are encoded by the genes PKBα, PKBβ and PKBγ in mammals, respectively. Akt is activated by phosphorylation at Thr^{308} by 3-phosphoinositide-dependent protein kinase 1 (PDK1), and also by phosphorylation within the C-terminus at Ser^{473} by PDK2 [45]. PI3K activates Akt, which transmits signals from cytokines, growth factors, and oncoproteins to multiple targets. Activated Akt could promote cell survival by inhibiting apoptosis through inactivation of several pro-apoptotic factors including Bcl-xL/Bcl-2-Associated Death (BAD), Forkhead transcription factors and caspase-9 [45].

Recently, PDGF-D has been shown to crosstalk with the PI3K/Akt pathway. It has been reported that the PDGF signaling pathways could be evaluated by detection of Tyr residue autophosphorylation of PDGF-activated PDGFR, and phospho-Akt of PI3K after PDGF-D treatment. The results suggest that PDGF-D is important to induce PDGFR-β autophosphorylation, and phosphorylation of Akt [44]. Ammoun et al. also reported that PDGF-D activated PDGFR-β and p-Akt in human Schwannoma cells [46]. Blocking PDGFR signaling using CT52923, a potent selective small molecule piperazinyl quinazoline kinase inhibitor of the PDGFR, also inhibited the phospho-Akt in glioblastoma cell lines [12]. Interestingly, we found that the total Akt expression was up-regulated in PDGF-D over-expressing PC3 (PC-3 PDGF-D) cells while p-Akt was reduced in PC-3 PDGF-D cells.

Moreover, LY294002, a PI3K inhibitor, reversed the rapamycin (a mTOR inhibitor)-induced activation of Akt in PC3 PDGF-D cells, suggesting that prolonged exposure of cells to PDGF-D activates the mTOR pathway, which, in turn, represses Akt activity in PC3 PDGF-D cells through a PI3K-dependent manner [7]. These results suggest that prolonged exposure of cells to PDGF-D leads to hyperactivation of mTOR, which is responsible for inactivation of Akt [7]. Further research toward exploration of the molecular mechanisms by which PDGF-D regulates PI3K/Akt requires immediate attention.

5.1.2. Mammalian target of rapamycin signaling and its role in the regulation of PDGF-D

The mTOR pathway has been reported to crosstalk with the PDGF-D pathway [7]. mTOR regulates translation rates and cell proliferation mainly by phosphorylating two major targets, the ribosomal protein S6 kinases (S6K1 and S6K2) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). S6K1 directly phosphorylates the 40S ribosomal protein S6, and then promotes ribosome biogenesis. 4E-BP1 is released from eIF4E after phosphorylation, allowing eIF4E to assemble with other translation initiation factors to initiate cap-dependent translation [45]. mTOR exists in two distinct complexes (mTORC1 and mTORC2): mTORC1 consists of mTOR, G-protein β-subunit-like protein (GβL), raptor and proline-rich Akt substrate of 40 kDa (PRAS40) protein, whereas mTORC2 contains mTOR, GβL, rictor and stress-activated protein kinase interacting protein 1 (SIN1). The raptor-containing complex is sensitive to rapamycin and regulates cell proliferation through phosphorylating S6K and 4E-BP1, while the rictor-containing complex is not sensitive to rapamycin [47].

Recently, the mTOR protein kinase has emerged as a critical player for controlling many cellular processes, such as cell growth and migration, by receiving stimulatory signals from PDGF-D. We have found that prostate cancer PC3 cells transfected with PDGF-D exhibit a rapid growth rate and increased invasion *in vitro*, which were associated with a high level of mTOR activity [7]. Specifically, PDGF-D markedly increased the levels of p-mTOR, p-4E-BP1, and p-S6K in prostate cancer cells. Moreover, we found that rapamycin increased phosphorylation of Akt in PC3 PDGF-D cells [7], suggesting that inactivation of Akt is attributed to a negative feedback regulation mediated by the mTOR pathway. However, the molecular mechanism(s) by which PDGF-D may be involved in the regulation of mTOR pathway or *vice versa* remains to be elucidated.

5.1.3. Nuclear factor-κB signaling and its role in PDGF-D signaling

Recently, several studies have shown that PDGF-D regulates the NF-κB pathway [8]. NF-κB plays important roles in the control of cell growth, differentiation, apoptosis and stress response. Without stimulation, NF-κB is sequestered in the cytoplasm through tight association with the specific inhibitory proteins IκB. Many stimuli can activate NF-κB, which leads to IKK (IκB kinase)-dependent phosphorylation and subsequent proteasome-mediated degradation of IκB proteins. Activated NF-κB, migrates into the nucleus and binds to the NF-κB-specific DNA-binding sites or interact with other transcription factors, and thus regulates gene transcription, including cytokines, chemokines, and anti-apoptotic factors [48]. A key regulatory step in the NF-κB pathway is the activation of IKK complex in which catalysis is thought to be via kinases, including IKKα and IKKβ, which directly phosphorylate IκB proteins. It has been reported that the interplay between the NF-κB and PDGF-D is biologically important. For example, we found that over-expression of PDGF-D in prostate cancer cells increased NF-κB DNA-binding activity [6]. Moreover, studies from our lab also showed that the down-regulation of PDGF-D in pancreatic cancer cells leads to the inactivation of NF-κB DNA-binding activity and, in turn, down-regulates the expression of its target genes, such as *MMP-9* and *VEGF* [8]. Recently, we also found that down-regulation of PDGF-D in breast cancer cells inhibited the NF-κB DNA-binding activity (unpublished data). Further in-depth studies are

needed to ascertain the precise molecular regulation of PDGF-D and NF-κB, and their crosstalks for elucidating the role of PDGF-D in cell growth, invasion and angiogenesis of cancer cells.

5.1.4. Notch signaling and its crosstalks with PDGF-D signaling

To date, in mammals, the Notch family of trans-membrane receptors consists of four members: Notch-1–4. Mammals also express Notch ligands and five such members have been found: Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-like 4), Jagged-1 and Jagged-2 [49]. Although these four Notch receptors show subtle differences in their extracellular and cytoplasmic domains, they are very similar. The extracellular domain of Notch possesses EGF-like repeats, which participate in ligand binding. The amino-terminal EGF-like repeats are followed by cysteine-rich Notch Lin12 repeats (N/Lin12) that prevent signaling in the absence of the ligand. The cytoplasmic region of Notch conveys the signal to the nucleus; it contains a RBP-J (Recombination Signal-Binding Protein 1 for J-kappa)-association molecule (RAM) domain, ankyrin repeats, TAD (trans-activation domain), NLS (nuclear localization signals) and a region rich in PEST (proline, glutamine, serine and threonine residues) sequence [50–52]. Notch signaling is initiated when Notch ligand binds to an adjacent Notch receptor between two neighboring cells. Upon activation, Notch is cleaved, releasing the intracellular domain of the Notch (ICN) which occurs through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase complex (comprised of presenilin-1/2, nicastrin, Pen-2, and Aph-1), resulting in the translocation of ICN into the nucleus for transcriptional activation of Notch target genes [53].

The growing body of literature strongly suggests the crosstalk between PDGF-D signaling and Notch pathway in cancer. We found that down-regulation of PDGF-D leads to the inactivation of Notch-1 in pancreatic cancer cells [8]. Therefore, the inactivation of PDGF-D-mediated cell invasion and angiogenesis could in part be due to inactivation of Notch-1 activity [8]. Moreover, we found that down-regulation of PDGF-D inhibited the Notch-1 expression in breast cancer cells (unpublished data). Recently, we reported that PDGF-D signaling contributes to epithelial to mesenchymal (EMT) phenotype which regulates cancer cell invasion and angiogenesis [6]. We also found that the expression of both mRNA and protein levels of Notch 1–4, Dll-1, Dll-3, Dll-4, Jagged-2 as well as Notch downstream targets, such as Hes and Hey, were significantly higher in PC3 PDGF-D cells (unpublished data). More importantly, we found that Notch-1 could be one of many miR-200b targets because over-expression of miR-200b significantly inhibited Notch-1 expression (unpublished data). However, how the miR-200b regulates Notch gene expression will certainly require further in-depth investigations.

5.1.5. Mitogen-activated protein kinase (MAPK) in relation to PDGF-D signaling

The MAPK has been known to play critical roles in controlling the balance between cell survival and apoptosis. A variety of cellular stimuli can activate the MAPK cascade, leading to the regulation of cell growth and apoptosis. It has been reported that MAPK pathway consists of three-tiered kinase core where a MAP3K activates MAP2K that in turn activates MAPK, resulting in the regulation of cell growth, and cell survival [54]. It has been well documented that activation of MAPK are also linked with cancer invasion, angiogenesis, and metastasis. Recent evidences suggest that PDGF-D is an effector of MAPK signaling. PDGF-D induced PDGFR-β autophosphorylation, and phosphorylation of JNK, p38 MAPK [44]. Blocking PDGFR signaling using PDGFR inhibitor CT52923 also inhibited the phospho-MAPK in glioblastoma cell lines [12]. However, more studies are required to understand how PDGF-D regulates MAPK signaling pathway in human malignancies.

5.1.6. Extracellular signal-regulated kinase (ERK) and its role in PDGF-D signaling

ERK activities were found to be up-regulated in many human tumors, and higher activity in tumors was associated with a poor prognosis, suggesting the crucial role of ERK in tumor progression [55]. ERK family has ERK1 and ERK2, which are members of the MAPK super family that can mediate cell proliferation and could regulate apoptosis. It has been well documented that multiple phosphatases (such as MAPK phosphatases) inactivate ERKs, suggesting that the duration and extent of ERK activation is tightly controlled by maintaining the balanced activities of (MAPK/ERK) kinase MEKs and respective phosphatases [56]. Recently, it has been found that PDGF-D can activate the ERK, suggesting that ERK is a major PDGF-D effector [44,46]. Ammoun et al. reported that PDGF-D activates PDGFR-β and ERK1/2 in human Schwannoma cells. PDGF-D promoted schwannoma cell proliferation, while inhibition of MAPK/ERK kinase 1/2 (MEK1/2) decreased PDGF-D-mediated proliferation, suggesting that ERK1/2 pathway is involved in this process [46]. Very interestingly, PDGF-D has different pathways to activate ERK1/2 that is localized to different intracellular compartments. The p-ERK1/2 pathway at 42/44 kDa (cytosol) uses PI3K-PKC-Src-c-Raf-dependent pathway and crosstalk with Akt at the level of PI3K, whereas PDGF-DD-mediated ERK1/2 activation at the 300-kDa level engages c-Raf, PKC, src, and PAK and localizes to the different cellular compartments than active ERK1/2 42/44 kDa [46]. These results suggest cooperation between PI3K, PKC, and Src in PDGF-D-mediated ERK1/2 activation. These results also suggest that a combined therapy targeting different pathways including PDGF-D pathway might be appropriate for treating schwannoma. The results from our laboratory also showed that condition medium form PC3-PDGF-D and LNCaP-PDGF-D, but not PC3 and LNCaP cells, induced an increase in ERK1/2 activation in NIH 3T3 cells, indicating that PDGF-D can regulate the ERK activation [26]. However, it remains to be determined how PDGF-D regulates the ERK activation in human cancers and whether inhibition of both PDGF-D and ERK signaling could be superior than targeting only PDGF-D or ERK in killing cancer cells.

5.2. Regulation of epithelial–mesenchymal transition

5.2.1. E-cadherin and Snail expression and their role in PDGF-D signaling

In recent years, PDGF-D has been found to play important roles in the acquisition of epithelial–mesenchymal transition (EMT) phenotype of cancer cell [6,57]. It is now widely accepted that epithelial cells can convert into mesenchymal cells by a fundamental process that is defined as EMT. Epithelial cells undergo remarkable morphologic changes from epithelial cobblestone phenotype to elongated fibroblastic phenotype (mesenchymal phenotype) and during the acquisition of EMT characteristics, cells lose epithelial cell–cell junction, actin cytoskeleton reorganization and the expression of proteins that promote cell–cell contact such as E-cadherin and γ-catenin [58]. Cells gain the expression of mesenchymal markers such as vimentin, α-smooth muscle actin (SMA), fibronectin, fibrillar collagen (types I and III), fibroblast-specific protein-1 and N-cadherin. EMT-type cells also showed increased activity of matrix metalloproteinases (MMPs) like MMP-2, MMP-3 and MMP-9, leading to increased migration and invasion [58]. A number of factors, including the zinc finger Snail homologues (Snail1, Snail2/Slug, and Snail3) and several basic helix-loop-helix factors such as Twist, zinc finger E-box binding homeobox 1 (ZEB1), ZEB2/SIP1, and TCF3/E47/E12, have emerged as potent EMT drivers during normal development and cancer [58], suggesting that these molecules are important regulator of EMT.

During the acquisition of EMT phenotype, the E-cadherin down-regulation is the crucial step in reducing cell–cell adhesion, leading to destabilization of the epithelial architecture. Indeed, we found that over-expression of PDGF-D in prostate cancer cells resulted in a significant induction of EMT concomitant with the loss of E-cadherin

[6]. We also found that the treatment of PC-3 prostate cancer cells with epithelial characteristics by purified PDGF-D protein resulted in a significantly decreased expression of E-cadherin at both the mRNA and protein levels [57]. It is known that a central role in E-cadherin gene repression is attributed to the Snail that is activated and triggers EMT. Snail binds to the two E-boxes of human E-cadherin promoter and then function as a repressor of E-cadherin gene. Snail is activated by most of the signaling pathways that are known to trigger EMT phenotype. As expected, PDGF-D was found to promote EMT through Snail because we found that the treatment of PC-3 prostate cancer cells (epithelial-like cells) by purified PDGF-D protein resulted in increased expression of Snail2 associated with induction of EMT characteristics. Moreover, Snail2 expression was dramatically up-regulated in PC3 PDGF-D cells, indicating that PDGF-D triggers EMT phenotype, which occurs in part through up-regulation of Snail2 [57]. It is important to note that PDGF-D also significantly increased the expression of ZEB1, ZEB2, N-cadherin, and vimentin [57], with concomitant loss of E-cadherin; however, further in-depth mechanistic studies are required for understanding how PDGF-D regulates E-cadherin and the processes of EMT phenotype.

5.2.2. The regulatory role of microRNAs (miRNAs) with respect to PDGF-D signaling

In recent years, PDGF-D has been found to crosstalk with miRNA [57]. It has been well documented that miRNAs work as integral players in cancer biology. The miRNAs elicit their regulatory effects in post-transcriptional regulation by binding to the 3′ untranslated region (3′ UTR) of target messenger RNA (mRNA) [59]. Either perfect or near perfect complimentary base pairing results in the degradation of the mRNA, while partial base pairing leads to translational inhibition of functional proteins. It is known that miRNAs are key players in human cancer because miRNAs are involved in the biological processes of cell proliferation and apoptosis, which are critically involved in the development and progression of human malignancies [60,61]. Recent studies also suggest that miRNAs could have diagnostic, prognostic, and therapeutic value [62]. Although, studies elucidating the role of miRNAs in cancer have exploded in recent years, it is still not clear how specific miRNA is regulated and what specific genes are the real targets of specific miRNA in tumor progression. Once that becomes clear then strategies to inactivate of active specific miRNA would become newer targeted approaches for the prevention of tumor progression and/or treatment of most human malignancies.

Recently, PDGF-D has been reported to crosstalk with miRNA-200 [57]. The microRNA-200 family has five members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429. Recently many studies have shown that the miR-200 family regulates EMT by targeting ZEB1 and ZEB2 [63,64]. We found that the treatment of cells with purified PDGF-D protein resulted in significant repression of the expression of miR-200a, miR-200b, and miR-200c. Moreover, we found that PDGF-D over-expression led to the acquisition of EMT phenotype in PC-3 prostate cells consistent with the loss of miR-200 expression, and that the re-expression of miR-200b in PC3 PDGF-D cells led to the reversal of the EMT phenotype, which was associated with the down-regulation of ZEB1, ZEB2, and Snail2 expression [57]. Moreover, transfection of PC3 PDGF-D cells with miR-200b inhibited cell migration and invasion with concomitant repression of cell adhesion to the culture surface and cell detachment, suggesting that PDGF-D is indeed responsible for the induction of the EMT phenotype in PC3 cells, which is in part mediated via down-regulation of miR-200 expression, and the regulation of its target genes [57]. Furthermore, we also found that PDGF-D triggers the EMT partly through down-regulation of let-7 in prostate cancer cells (unpublished data). Clearly, specific role of selected miRNA in PDGF-D signaling pathway has begun to be explored, and therefore further studies are needed in elucidating the role of specific miRNA in the regulation of PDGF-D mediated signaling pathway.

5.3. Regulation of cell invasion, angiogenesis, and metastasis

5.3.1. The role of matrix metalloproteinases (MMPs) in PDGF-D signaling

Tumor metastasis occurs through several steps requiring cell migration, invasion, degradation of basement membranes and the stromal extracellular matrix, ultimately leading to tumor cell metastasis, which is accomplished by many proteolytic enzymes including MMPs. The MMPs are a family of related enzymes which are capable of degrading extracellular matrix, and this process is crucial during tumor cell invasion, angiogenesis and metastasis. MMPs have also been implicated in the acquisition of EMT, a "hallmark" of tumor progression and metastasis. Evidence is emerging showing that members of the MMP family can serve not only as potential markers for diagnosis, prognosis, and early detection, but also as indicators of tumor recurrence, metastatic spread, and response to therapy for human cancers [65]. Among these MMPs, MMP-9 and MMP-2 have been found to be important factors in facilitating invasion and metastases in human cancers because they are directly linked with angiogenesis and degradation of the basement membrane collagen [65].

It has been reported that MMP-9 expression is regulated by PDGF-D in several kinds of human cancers. Xu et al. found that over-expression of PDGF-D in renal cancer SN12-C cells promoted tumor growth, angiogenesis and metastasis due to increased expression of MMP-9 and angiopoietin-1 in an orthotopic mouse model [27]. We also found that down-regulation of PDGF-D in pancreatic cancer cells leads to the inhibition of MMP-9 expression and MMP-9 activation [8]. Recently, we found that down-regulation of PDGF-D also decreased the expression of MMP-9 in breast cancer cells (unpublished data). More recently, Zhao et al. reported that inhibition of PDGF-D leads to decreased cell invasion and angiogenesis in gastric cancer through MMP-9 and MMP-2 [66]. In order to better understand the precise role of PDGF-D and its interrelationship with MMPs requires in-depth investigation.

5.3.2. Vascular endothelial cell growth factor (VEGF) and its relationship with PDGF-D signaling

Studies have shown that VEGF is very crucial for the induction of angiogenic processes because VEGF regulates most of the steps in the angiogenesis including migration, invasion, and tube formation of endothelial cells. In addition, studies have shown a trend towards an association between the expression of VEGF and migration, invasion and distant metastasis [67]. It has been reported that PDGF-D regulates VEGF signaling in various cancer cell types. Li et al. found that PDGF-D is a potent transforming growth factor for NIH/3T3 cells, and the transformed cells displayed increased proliferation rate, induced tumors in nude mice, and up-regulated VEGF [68]. We also found that down-regulation of PDGF-D in pancreatic cancer cells led to the inhibition of VEGF secretion [8]. Moreover, conditioned medium from PDGF-D siRNA-transfected cells showed reduced levels of VEGF, resulting in the inhibition of angiogenesis which was assessed by the tube formation of human umbilical vein endothelial cells (HUVECs), suggesting that down-regulation of PDGF-D is responsible for the inhibition of angiogenesis [8]. In contrast, condition medium from PDGF-D over-expressing PC3 cells induced tube formation of HUVECs [6]. Very recently, Zhao et al. found that inhibition of PDGF-D leads to decreased cell invasion and angiogenesis in gastric cancer partly through the regulation of VEGF [66]. However, the molecular mechanism(s) by which VEGF is regulated by PDGF-D or *vice versa* is poorly understood, and thus in-depth investigation in this area is urgently needed.

5.3.3. The crosstalks of other effectors in the regulation of PDGF-D

Several recent studies have shown that PDGF-D regulates many other effectors, such as Bcl-2, Cyclin D1, and β-catenin, which have not been discussed above. For example, we found a dramatic increase in the levels of Bcl-2 expression in PDGF-D over-expressing prostate cancer cells, and the regulation of Bcl-2 expression by PDGF-D was shown to play an important role in PDGF-D-mediated acquisition of EMT [6,7]. Conversely, the down-regulation of PDGF-D was able to decrease the expression of Bcl-2 in pancreatic cancer cell [8]. In addition, we found that PDGF-D can up-regulate the expression of poly(ADPribose) polymerase-1 (PARP-1) in prostate cancer cells [6]. Further studies by Zhao et al. have shown that down-regulation of PDGF-D inhibited Cyclin D1 and β-catenin in gastric cancer [66], and similar results were reported in pancreatic and prostate cancer [8,57]. Since PDGF-D signaling is an emerging area of research in multiple human malignancies, there is no doubt that we will be witnessing the role of additional effectors of PDGF-D signaling in years to come.

6. Conclusion and overall perspectives

Given the importance of PDGF-D in tumor cell growth, migration, invasion, angiogenesis and metastasis and its crosstalks with many signaling pathways in human malignancies (Fig. 2), significant attention
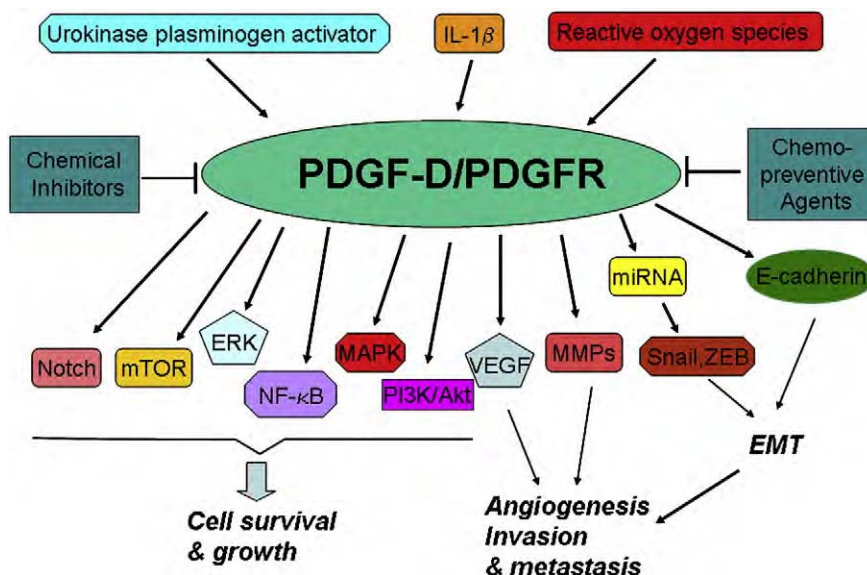

Fig. 2. Diagram of PDGF-D crosstalks with other pathways. ERK: extracellular signal-regulated kinase; IL-1β: proinflammatory cytokine interleukin-1β; MAPK: mitogen-activated protein kinase; MMPs: matrix metalloproteinases; mTOR: mammalian target of rapamycin; NF-κB: nuclear factor-κB; PI3K: phosphatidylinositol 3-kinase; ROS: reactive oxygen species; uPA: urokinase-type plasminogen activator; VEGF: vascular endothelial growth factor. EMT: epithelial–mesenchymal transition.

has been paid in recent past toward the development of clinically useful antagonists of PDGF signaling. PDGF-D displays an oncogenic activity specifically through binding to and activating its cognate receptor PDGFR-β, suggesting that the inactivation of PDGF-D/PDGFR signaling by novel approaches is likely to have a significant impact in cancer therapy. Several small molecule tyrosine kinase inhibitors that block the PDGF receptor have been developed. For example, CT52923, one of PDGFR antagonists, inhibited survival and/or mitogenic pathways in the glioblastoma cell lines and prevented glioma formation in a nude mouse xenograft model [12]. Imatinib (STI571 or Gleveec), which is a selective tyrosine kinase inhibitor especially for the inhibition of PDGFR, inhibited the cell growth and invasion in human breast cancer cell lines [69]. The combination of imatinib with chemotherapeutic agent paclitaxel or gemcitabine led to a further tumor growth inhibition in prostate cancer and malignant mesothelioma, suggesting that imatinib enhances the therapeutic response to chemotherapeutic agents [70,71]. CR002, a humanized monoclonal PDGF-D antibody, has been shown to be safe in a phase I study, and CR002 was able to reduce glomerular and secondary tubulointerstitial damage [72]; however, CR002 has not yet been tried for its effects in human cancer.

To our knowledge, there is no report regarding the small chemical inhibitors of PDGF-D but we are confident that such search must be an active area of research. Interestingly, we found that 3,3′-Diindolyl-methane (DIM, a well known chemopreventive agent) significantly inhibited the expression and activation of PDGF-D in prostate cancer cells [7]. Our results suggest that DIM could serve as a novel and efficient chemopreventive and/or therapeutic agent by inactivation of PDGF-D in prostate cancer cells especially because DIM was found to be non-toxic in most human studies. We and other investigators have demonstrated that increased expression of PDGF-D and its receptor is detected in many human cancer cells and tissues. More importantly, PDGF-D plays important roles in almost all aspects of cancer biology, such as proliferation, apoptosis, migration, invasion, angiogenesis and metastasis; however, further in-depth studies including mechanistic *in vitro* studies and *in vivo* animal experiments are needed to fully understand and appreciate the roles of PDGF-D in tumor progression. We believe that this article would be able to stimulate or promote further research in this field toward the development of novel approaches by which PDGF-D signaling could be targeted for the inhibition of tumor progression and/or therapy for most human malignancies.

Acknowledgements

References

[1] J. Andrae, R. Gallini, C. Betsholtz, Role of platelet-derived growth factors in physiology and medicine, Genes Dev. 22 (2008) 1276–1312.

[2] E. Bergsten, M. Uutela, X. Li, K. Pietras, A. Ostman, C.H. Heldin, K. Alitalo, U. Eriksson, PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor, Nat. Cell Biol. 3 (2001) 512–516.

[3] W.J. LaRochelle, M. Jeffers, W.F. McDonald, R.A. Chillakuru, N.A. Giese, N.A. Lokker, C. Sullivan, F.L. Boldog, M. Yang, C. Vernet, C.E. Burgess, E. Fernandes, L.L. Deegler, B. Rittman, J. Shimkets, R.A. Shimkets, J.M. Rothberg, H.S. Lichenstein, PDGF-D, a new protease-activated growth factor, Nat. Cell Biol. 3 (2001) 517–521.

[4] X. Li, U. Eriksson, Novel PDGF family members: PDGF-C and PDGF-D, Cytokine Growth Factor Rev. 14 (2003) 91–98.

[5] Z. Wang, D. Kong, Y. Li, F.H. Sarkar, PDGF-D signaling: a novel target in cancer therapy, Curr. Drug Targets 10 (2009) 38–41.

[6] D. Kong, Z. Wang, S.H. Sarkar, Y. Li, S. Banerjee, A. Saliganan, H.R. Kim, M.L. Cher, F.H. Sarkar, Platelet-derived growth factor-D overexpression contributes to epithelial–mesenchymal transition of PC3 prostate cancer cells, Stem Cells 26 (2008) 1425–1435.

[7] D. Kong, S. Banerjee, W. Huang, Y. Li, Z. Wang, H.R. Kim, F.H. Sarkar, Mammalian target of rapamycin repression by 3, 3′-diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells, Cancer Res. 68 (2008) 1927–1934.

[8] Z. Wang, D. Kong, S. Banerjee, Y. Li, N.V. Adsay, J. Abbruzzese, F.H. Sarkar, Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling, Cancer Res. 67 (2007) 11377–11385.

[9] L.J. Reigstad, J.E. Varhaug, J.R. Lillehaug, Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family, FEBS J. 272 (2005) 5723–5741.

[10] L. Fredriksson, H. Li, U. Eriksson, The PDGF family: four gene products form five dimeric isoforms, Cytokine Growth Factor Rev. 15 (2004) 197–204.

[11] K. Breitkopf, C. Roeyen, I. Sawitza, L. Wickert, J. Floege, A.M. Gressner, Expression patterns of PDGF-A, -B, -C and -D and the PDGF-receptors alpha and beta in activated rat hepatic stellate cells (HSC), Cytokine 31 (2005) 349–357.

[12] N.A. Lokker, C.M. Sullivan, S.J. Hollenbach, M.A. Israel, N.A. Giese, Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors, Cancer Res. 62 (2002) 3729–3735.

[13] E.A. Maher, F.B. Furnari, R.M. Bachoo, D.H. Rowitch, D.N. Louis, W.K. Cavenee, R.A. DePinho, Malignant glioma: genetics and biology of a grave matter, Genes Dev. 15 (2001) 1311–1333.

[14] W.J. LaRochelle, M. Jeffers, J.R. Corvalan, X.C. Jia, X. Feng, S. Vanegas, J.D. Vickroy, X.D. Yang, F. Chen, G. Gazit, J. Mayotte, J. Macaluso, B. Rittman, F. Wu, M. Dhanabal, J. Herrmann, H.S. Lichenstein, Platelet-derived growth factor D: tumorigenicity in mice and dysregulated expression in human cancer, Cancer Res. 62 (2002) 2468–2473.

[15] R. Ross, Platelet-derived growth factor, Annu. Rev. Med. 38 (1987) 71–79.

[16] C.H. Heldin, B. Westermark, Platelet-derived growth factor: three isoforms and two receptor types, Trends Genet. 5 (1989) 108–111.

[17] C.H. Heldin, B. Westermark, Mechanism of action and in vivo role of platelet-derived growth factor, Physiol. Rev. 79 (1999) 1283–1316.

[18] L. Claesson-Welsh, Mechanism of action of platelet-derived growth factor, Int. J. Biochem. Cell Biol. 28 (1996) 373–385.

[19] C.H. Heldin, A. Hammacher, M. Nister, B. Westermark, Structural and functional aspects of platelet-derived growth factor, Br. J. Cancer 57 (1988) 591–593.

[20] D. George, Platelet-derived growth factor receptors: a therapeutic target in solid tumors, Semin. Oncol. 28 (2001) 27–33.

[21] J. Yu, C. Ustach, H.R. Kim, Platelet-derived growth factor signaling and human cancer, J. Biochem. Mol. Biol. 36 (2003) 49–59.

[22] D. George, Targeting PDGF receptors in cancer—rationales and proof of concept clinical trials, Adv. Exp. Med. Biol. 532 (2003) 141–151.

[23] M. Li, V. Jendrossek, C. Belka, The role of PDGF in radiation oncology, Radiat. Oncol. 2 (2007) 5.

[24] H. Lei, A. Kazlauskas, Focus on molecules: platelet-derived growth factor C, PDGF-C, Exp. Eye Res. 86 (2008) 711–712.

[25] C.V. Ustach, M.E. Taube, N.J. Hurst Jr., S. Bhagat, R.D. Bonfil, M.L. Cher, L. Schuger, H.R. Kim, A potential oncogenic activity of platelet-derived growth factor d in prostate cancer progression, Cancer Res. 64 (2004) 1722–1729.

[26] C.V. Ustach, H.R. Kim, Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells, Mol. Cell Biol. 25 (2005) 6279–6288.

[27] L. Xu, R. Tong, D.M. Cochran, R.K. Jain, Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model, Cancer Res. 65 (2005) 5711–5719.

[28] L.M. Khachigian, A.J. Williams, T. Collins, Interplay of Sp1 and Egr-1 in the proximal platelet-derived growth factor A-chain promoter in cultured vascular endothelial cells, J. Biol. Chem. 270 (1995) 27679–27686.

[29] V.C. Midgley, L.M. Khachigian, Fibroblast growth factor-2 induction of platelet-derived growth factor-C chain transcription in vascular smooth muscle cells is ERK-dependent but not JNK-dependent and mediated by Egr-1 1, J. Biol. Chem. 279 (2004) 40289–40295.

[30] L.M. Khachigian, V. Lindner, A.J. Williams, T. Collins, Egr-1-induced endothelial gene expression: a common theme in vascular injury, Science 271 (1996) 1427–1431.

[31] M.Y. Liu, M. Eyries, C. Zhang, F.S. Santiago, L.M. Khachigian, Inducible platelet-derived growth factor D-chain expression by angiotensin II and hydrogen peroxide involves transcriptional regulation by Ets-1 and Sp1, Blood 107 (2006) 2322–2329.

[32] N.Y. Tan, V.C. Midgley, M.M. Kavurma, F.S. Santiago, X. Luo, R. Peden, R.G. Fahmy, M.C. Berndt, M.P. Molloy, L.M. Khachigian, Angiotensin II-inducible platelet-derived growth factor-D transcription requires specific Ser/Thr residues in the second zinc finger region of Sp1, Circ. Res. 102 (2008) e38–e51.

[33] M.Y. Liu, L.M. Khachigian, Histone deacetylase-1 is enriched at the platelet-derived growth factor-D promoter in response to interleukin-1beta and forms a cytokine-inducible gene-silencing complex with NF-kappab p65 and interferon regulatory factor-1, J. Biol. Chem. 284 (2009) 35101–35112.

[34] K. Dass, A. Ahmad, A.S. Azmi, S.H. Sarkar, F.H. Sarkar, Evolving role of uPA/uPAR system in human cancers, Cancer Treat. Rev. 34 (2008) 122–136.

[35] A. Ahmad, D. Kong, Z. Wang, S.H. Sarkar, S. Banerjee, F.H. Sarkar, Down-regulation of uPA and uPAR by 3, 3′-diindolylmethane contributes to the inhibition of cell growth and migration of breast cancer cells, J. Cell Biochem. 108 (2009) 916–925.

[36] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8 (2009) 579–591.

[37] Z. Wang, Y. Li, F.H. Sarkar, Signaling mechanism(s) of reactive oxygen species in epithelial–mesenchymal transition reminiscent of cancer stem cells in tumor progression, Curr. Stem Cell Res. Ther. 5 (1) (Mar 2010) 74–80.

[38] M. Sundaresan, Z.X. Yu, V.J. Ferrans, K. Irani, T. Finkel, Requirement for generation of H2O2 for platelet-derived growth factor signal transduction, Science 270 (1995) 296–299.

[39] H. Lei, A. Kazlauskas, Growth factors outside of the platelet-derived growth factor (PDGF) family employ reactive oxygen species/Src family kinases to activate PDGF receptor alpha and thereby promote proliferation and survival of cells, J. Biol. Chem. 284 (2009) 6329–6336.

[40] H. Shimizu, Y. Hirose, F. Nishijima, Y. Tsubakihara, H. Miyazaki, ROS and PDGF-beta [corrected] receptors are critically involved in indoxyl sulfate actions that promote vascular smooth muscle cell proliferation and migration, Am. J. Physiol. Cell Physiol. 297 (2009) C389–C396.

[41] J.M. Ricono, B. Wagner, Y. Gorin, A. Arar, A. Kazlauskas, G.G. Choudhury, H.E. Abboud, PDGF receptor-{beta} modulates metanephric mesenchyme chemotaxis induced by PDGF AA, Am. J. Physiol. Ren. Physiol. 296 (2009) F406–F417.

[42] A. Ahmad, S. Banerjee, Z. Wang, D. Kong, A.P. Majumdar, F.H. Sarkar, Aging and inflammation: etiological culprits of cancer, Curr. Aging Sci. 2 (2009) 174–186.

[43] R. Li, A. Maminishkis, F.E. Wang, S.S. Miller, PDGF-C and -D induced proliferation/migration of human RPE is abolished by inflammatory cytokines, Invest. Ophthalmol. Vis. Sci. 48 (2007) 5722–5732.

[44] E. Borkham-Kamphorst, C.R. van Roeyen, T. Ostendorf, J. Floege, A.M. Gressner, R. Weiskirchen, Pro-fibrogenic potential of PDGF-D in liver fibrosis, J. Hepatol. 46 (2007) 1064–1074.

[45] P. Liu, H. Cheng, T.M. Roberts, J.J. Zhao, Targeting the phosphoinositide 3-kinase pathway in cancer, Nat. Rev. Drug Discov. 8 (2009) 627–644.

[46] S. Ammoun, C. Flaiz, N. Ristic, J. Schuldt, C.O. Hanemann, Dissecting and targeting the growth factor-dependent and growth factor-independent extracellular signal-regulated kinase pathway in human schwannoma, Cancer Res. 68 (2008) 5236–5245.

[47] J.A. Engelman, Targeting PI3K signalling in cancer: opportunities, challenges and limitations, Nat. Rev. Cancer 9 (2009) 550–562.

[48] F.H. Sarkar, Y. Li, Z. Wang, D. Kong, NF-kappaB signaling pathway and its therapeutic implications in human diseases, Int. Rev. Immunol. 27 (2008) 293–319.

[49] Z. Wang, Y. Li, S. Banerjee, F.H. Sarkar, Exploitation of the Notch signaling pathway as a novel target for cancer therapy, Anticancer Res. 28 (2008) 3621–3630.

[50] Z. Wang, Y. Li, S. Banerjee, F.H. Sarkar, Emerging role of Notch in stem cells and cancer, Cancer Lett. 279 (2009) 8–12.

[51] L. Miele, H. Miao, B.J. Nickoloff, Notch signaling as a novel cancer therapeutic target, Curr. Cancer Drug Targets 6 (2006) 313–323.

[52] L. Miele, Notch signaling, Clin. Cancer Res. 12 (2006) 1074–1079.

[53] Z. Wang, Y. Zhang, Y. Li, S. Banerjee, J. Liao, F.H. Sarkar, Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells, Mol. Cancer Ther. 5 (2006) 483–493.

[54] J.S. Sebolt-Leopold, R. Herrera, Targeting the mitogen-activated protein kinase cascade to treat cancer, Nat. Rev. Cancer 4 (2004) 937–947.

[55] E.F. Wagner, A.R. Nebreda, Signal integration by JNK and p38 MAPK pathways in cancer development, Nat. Rev. Cancer 9 (2009) 537–549.

[56] Y. Mebratu, Y. Tesfaigzi, How ERK1/2 activation controls cell proliferation and cell death: is subcellular localization the answer? Cell Cycle 8 (2009) 1168–1175.

[57] D. Kong, Y. Li, Z. Wang, S. Banerjee, A. Ahmad, H.R. Kim, F.H. Sarkar, miR-200 regulates PDGF-D-mediated epithelial–mesenchymal transition, adhesion, and invasion of prostate cancer cells, Stem Cells 27 (2009) 1712–1721.

[58] J.P. Thiery, H. Acloque, R.Y. Huang, M.A. Nieto, Epithelial–mesenchymal transitions in development and disease, Cell 139 (2009) 871–890.

[59] C.M. Croce, Causes and consequences of microRNA dysregulation in cancer, Nat. Rev. Genet. 10 (2009) 704–714.

[60] M.V. Iorio, C.M. Croce, MicroRNAs in cancer: small molecules with a huge impact, J. Clin. Oncol. 27 (2009) 5848–5856.

[61] T.G. Vandenboom Ii, Y. Li, P.A. Philip, F.H. Sarkar, MicroRNA and cancer: tiny molecules with major implications 1262, Curr. Genomics 9 (2008) 97–109.

[62] B.D. Brown, L. Naldini, Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications, Nat. Rev. Genet. 10 (2009) 578–585.

[63] P.A. Gregory, A.G. Bert, E.L. Paterson, S.C. Barry, A. Tsykin, G. Farshid, M.A. Vadas, Y. Khew-Goodall, G.J. Goodall, The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1, Nat. Cell Biol. 10 (2008) 593–601.

[64] U. Wellner, J. Schubert, U.C. Burk, O. Schmalhofer, F. Zhu, A. Sonntag, B. Waldvogel, C. Vannier, D. Darling, H.A. zur, V.G. Brunton, J. Morton, O. Sansom, J. Schuler, M.P. Stemmler, C. Herzberger, U. Hopt, T. Keck, S. Brabletz, T. Brabletz, The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs, Nat. Cell Biol. 11 (2009) 1487–1495.

[65] R. Roy, J. Yang, M.A. Moses, Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer, J. Clin. Oncol. 27 (2009) 5287–5297.

[66] L. Zhao, C. Zhang, G. Liao, J. Long, RNAi-mediated inhibition of PDGF-D leads to decreased cell growth, invasion and angiogenesis in the SGC-7901 gastric cancer xenograft model, Cancer Biol. Ther. 9 (2009) 1–7.

[67] H.X. Chen, J.N. Cleck, Adverse effects of anticancer agents that target the VEGF pathway, Nat. Rev. Clin. Oncol. 6 (2009) 465–477.

[68] H. Li, L. Fredriksson, X. Li, U. Eriksson, PDGF-D is a potent transforming and angiogenic growth factor, Oncogene 22 (2003) 1501–1510.

[69] A.E. Roussidis, A.D. Theocharis, G.N. Tzanakakis, N.K. Karamanos, The importance of c-Kit and PDGF receptors as potential targets for molecular therapy in breast cancer, Curr. Med. Chem. 14 (2007) 735–743.

[70] P. Bertino, F. Piccardi, C. Porta, R. Favoni, M. Cilli, L. Mutti, G. Gaudino, Imatinib mesylate enhances therapeutic effects of gemcitabine in human malignant mesothelioma xenografts, Clin. Cancer Res. 14 (2008) 541–548.

[71] S.J. Kim, H. Uehara, S. Yazici, J.E. Busby, T. Nakamura, J. He, M. Maya, C. Logothetis, P. Mathew, X. Wang, K.A. Do, D. Fan, I.J. Fidler, Targeting platelet-derived growth factor receptor on endothelial cells of multidrug-resistant prostate cancer, J. Natl. Cancer Inst. 98 (2006) 783–793.

[72] P. Boor, A. Konieczny, L. Villa, U. Kunter, C.R. van Roeyen, W.J. LaRochelle, G. Smithson, S. Arrol, T. Ostendorf, J. Floege, PDGF-D inhibition by CR002 ameliorates tubulointerstitial fibrosis following experimental glomerulonephritis, Nephrol. Dial. Transplant. 22 (2007) 1323–1331.

Review

Targeting Notch signaling pathway to overcome drug resistance for cancer therapy

Zhiwei Wang *, Yiwei Li, Aamir Ahmad, Asfar S. Azmi, Sanjeev Banerjee, Dejuan Kong, Fazlul H. Sarkar *

Department of Pathology, Karmanos Cancer Institute, Wayne State University, School of Medicine, Detroit, MI 48201, USA

ARTICLE INFO

ABSTRACT

Chemotherapy is an important therapeutic strategy for cancer treatment and remains the mainstay for the management of human malignancies; however, chemotherapy fails to eliminate all tumor cells because of intrinsic or acquired drug resistance, which is the most common cause of tumor recurrence. Recently, emerging evidences suggest that Notch signaling pathway is one of the most important signaling pathways in drug-resistant tumor cells. Moreover, down-regulation of Notch pathway could induce drug sensitivity, leading to increased inhibition of cancer cell growth, invasion, and metastasis. This article will provide a brief overview of the published evidences in support of the roles of Notch in drug resistance and will further summarize how targeting Notch by "natural agents" could become a novel and safer approach for the improvement of tumor treatment by overcoming drug resistance.

© 2010 Elsevier B.V. All rights reserved.

Contents

1. Introduction

The Notch signaling pathway is a conserved ligand–receptor signaling pathway that plays critical mechanistic roles in cell proliferation, survival, apoptosis, and differentiation which affects the development and function of many organs [1]. Notch genes

* Corresponding authors. Tel.: +1 313 5768327; fax: +1 313 5768389.
  E-mail addresses: zhiweiwang@med.wayne.edu (Z. Wang),
fsarkar@med.wayne.edu (F.H. Sarkar).

encode single-pass transmembrane proteins which can be activated by interacting with a family of its ligands. To date, four Notch receptors have been identified in mammals, including human, such as Notch-1-4. The mammalian canonical ligands are designated as either Delta-like (Delta-like 1, Delta-like 3, and Delta-like 4) or Serrate-like ligands, known as Jagged-1 and Jagged-2 [2]. All four Notch receptors are very similar except subtle differences in their extracellular and cytoplasmic domains. The extracellular domains of Notch contain many repeated copies of an epidermal growth factor (EGF)-like motif, which are involved in ligand interaction. Both Notch-1 and Notch-2 proteins have 36 arranged repeats of EGF-like domain, whereas Notch-3 and Notch-4 contain 34 and 29 EGF-like repeats, respectively [3]. The amino-terminal EGF-like repeats are followed by cysteine-rich Notch Lin12 repeats (N/Lin12) that modulate interactions between the extracellular and the membrane-tethered intracellular domains. The cytoplasmic region of Notch contains a Recombination Signal-Binding Protein 1 for J-kappa (RBP-J)-association molecule (RAM) domain, ankyrin (ANK) repeats, nuclear localization signals (NLS), a trans-activation domain (TAD), and a region rich in proline, glutamine, serine, and threonine residues (PEST) sequence. It is well known that ANK repeats are necessary and sufficient for Notch activity. PEST sequence is involved in Notch protein turnover [4] and the cytoplasmic region of conveys the signal to the nucleus. Notch ligands have multiple EGF-like repeats in their extracellular domain

and a cysteine-rich region (CR) in Serrate which are absent in Delta. Jagged-1 and Jagged-2 have almost two-fold numbers of EGF-like repeats compared to Delta [4] (Fig. 1A).

Notch signaling is activated after ligand binding to an adjacent Notch receptor between two neighboring cells. Upon activation, Notch receptors undergo a series of proteolytic cleavages by the metalloprotease, tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase complex (comprised of presenilin-1/2, nicastrin, Pen-2, and Aph-1). The first cleavage is mediated by TACE, which leads to cleave the receptor in the extracellular domain. The released extracellular domain is then trans-endocytosed by the ligand-expressing cell. The second cleavage caused by the γ-secretase complex releases the Notch intracellular domain (NICD) into the cytoplasm, which can subsequently translocate into the nucleus because of the presence of nuclear localization signals located within it [5]. Therefore, inhibiting γ-secretase function would prevent the cleavage of the Notch receptor, blocking Notch signal transduction, and thus γ-secretase inhibitor (GSI) could be useful for the treatment of human malignancies [6]. Consistent with this rationale, GSI are now undergoing clinical trials (see website: clinicaltrials.gov). In the absence of NICD, transcription of Notch target genes is inhibited by a repressor complex mediated by the CSL (C protein binding factor 1/Suppressor of Hairless/Lag-1). When NICD is in the nucleus, it forms an active transcriptional complex due to displacing the histone
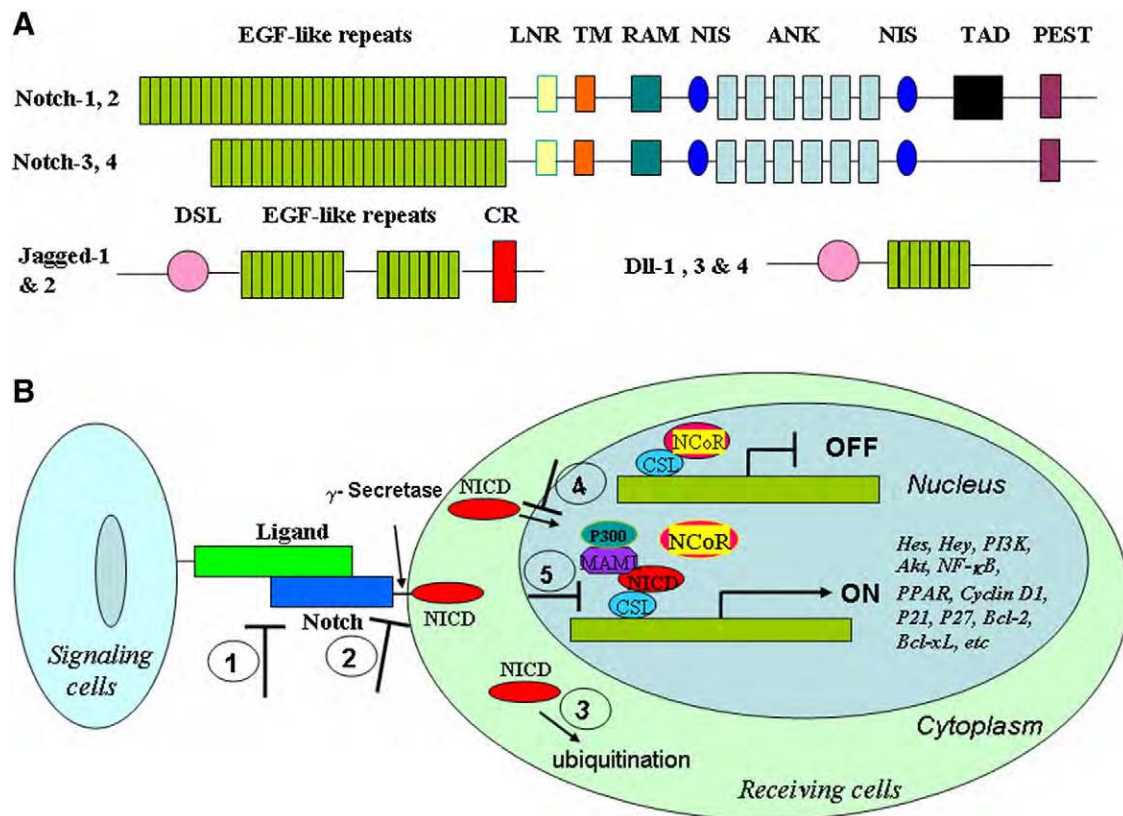

Fig. 1. (A) Structure of Notch receptors (1–4) and ligands (Jagged-1, 2, Dll-1, 3, 4). Both receptors and ligands contain multiple conserved domains. Notch is a single-pass transmembrane receptor. The extracellular domain contains EGF-like repeats and a cysteine-rich region. The intracellular domain contains the RAM domain, NLS, ANK, TAD, and PEST domain. Notch ligands have multiple EGF-like repeats in their extracellular domain and a CR in Jagged which are absent in Delta. (B) Schematic of Notch signaling. Notch signaling is activated after ligand binding to an adjacent Notch receptor between two neighboring cells. Upon activation, Notch receptors undergo a series of proteolytic cleavages by the metalloprotease, TACE, and γ-secretase complex. The cleavage releases the NICD into the cytoplasm, which can subsequently translocate into the nucleus. In the absence of NICD, transcription of Notch target genes is inhibited by a repressor complex mediated by the CSL. When NICD is in the nucleus, it forms an active transcriptional complex due to displacing the histone deacetylase–corepressor complex and recruiting the protein MAML1 and histone acetyltransferases to the CSL complex, leading to convert it from a transcriptional repressor into a transcription activator complex, leading to activation of Notch target genes. Diagram of putative therapeutic target in the Notch pathway. Notch signaling could be inhibited theoretically at many different levels. It is possible to (1) interfere with Notch–ligand interactions, (2) inhibit receptor activation, (3) promote Notch ubiquitination and degradation, (4) inhibit its translocation to the nuclear compartment, and (5) inhibit NICD nuclear complex formation. ANK: ankyrin repeat; DSL: delta-serrate-lag2; EGF: epidermal growth factor; LNR: Lin12/Notch repeats; NLS: nuclear localization signals; PEST: proline, glutamine, serine, threonine; RAM: RBP-J association molecule domain; TAD: transcriptional activator domain; TM: transmembrane domain. CSL: C protein binding factor 1/Suppressor of Hairless/Lag-1; NICD: Notch intracellular domain; TACE: tumor necrosis factor-α-converting enzyme; MAML1: mastermind-like 1.

deacetylase–corepressor complex and recruiting the protein mastermind-like 1 (MAML1) and histone acetyltransferases to the CSL complex, leading to convert it from a transcriptional repressor into a transcription activator complex [2] (Fig. 1B). A few Notch target genes have been identified, including Hes (Hairy enhance of split) family, Hey (Hairy/enhancer of spit related with YRPW motif), nuclear factor-kappa B (NF-κB), vascular growth factor receptor (VEGF), mammalian target of rapamycin (mTOR), cyclin D1, c-myc, p21, p27, Akt, etc., all of which have been well documented for their roles in tumor development and progression [7–10].

2. Notch in cancer development and progression

It has been well known that Notch signaling plays important roles in maintaining the balance involved in cell proliferation, survival, apoptosis, and differentiation which affects the development and function of many organs. Therefore, dysfunction of Notch prevents differentiation, ultimately guiding undifferentiated cells toward malignant transformation. Indeed, many observations suggest that alterations in Notch signaling are associated with many human cancers [11–17]. Moreover, Notch receptors and ligands have been found as prognostic markers in human cancers [18,19].

2.1. Notch functions as oncogene or tumor suppressor

Very interestingly, the function of Notch signaling in tumorigenesis could be either oncogenic or anti-proliferative, and the function could be context dependent. Notch signaling has been shown to be anti-proliferative in a limited number of tumor types, including skin cancer, human hepatocellular carcinoma, medullary thyroid, cervical cancer, and small cell lung cancer [20–24]. For example, Nicolas et al. [24] used a tissue-specific inducible gene-targeting approach to study the physiological role of the Notch-1 receptor in the mouse epidermis and the corneal epithelium of adult mice. They unexpectedly found that ablation of Notch-1 results in epidermal and corneal hyperplasia followed by the development of skin tumors and facilitated chemical-induced skin carcinogenesis through beta-catenin-mediated signaling. Recently, studies have also demonstrated that Notch-1 loss in epidermal keratinocytes promotes tumorigenesis by impairing skin-barrier integrity and creating a wound-like microenvironment in the skin. Using mice with a chimeric pattern of Notch-1 deletion, the authors have found that Notch-1 was insufficient to suppress tumor-promoting effect, and the tumor-promoting effect of Notch-1 loss involves a cross talk between barrier-defective epidermis and its stroma [25]. More recent findings obtained in melanoma and non-melanoma skin cancers show that Notch signaling has a dual action (either as an oncogene or as a tumor suppressor), depending on the tumor cell type and involving synchronous activation of other intracellular signaling mechanisms [26].

However, most of the studies have shown oncogenic function of Notch in many human carcinomas. Emerging evidence suggest that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands were found in breast, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin, and large-cell lymphomas and pancreatic cancer [8,9,15,27–30]. Notch signaling pathway has also been found to cross talk with multiple oncogenic signaling pathways, such as NF-κB, Akt, Sonic hedgehog (Shh), mTOR, Ras, Wnt, estrogen receptor (ER), androgen receptor (AR), epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) [31–36]. Thus it is believed that the cross talk between Notch and other signaling pathways may play critical roles in tumor aggressiveness. From the literature, Notch may act either as a tumor suppressor or tumor promoter depending on the cell type and tissue context, suggesting the complexity of Notch signaling pathways [26]. The functions of Notch signaling have recently been reviewed [7,10,16,23,27,37–40], and thus the readers who are interested in learning more details on the functions of Notch signaling pathway could also consult well-published review articles because the focus of the current article is restricted to overcoming drug resistance.

2.2. Notch as diagnostic and prognostic markers in human cancers

Notch signaling pathway has been shown to play a role in cancer patient survival. Patients with tumors expressing high levels of Jagged-1 or Notch-1 had a significantly poorer overall survival compared with patients expressing low levels of these genes. Jagged-1 was also found to be highly expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues [41]. Furthermore, high Jagged-1 expression in a subset of clinically localized tumors was significantly associated with recurrence, suggesting that Jagged-1 may be a useful marker in distinguishing indolent vs. aggressive prostate carcinomas [41]. Recently, high level co-expression of Jagged-1 and Notch-1 has been observed in human breast cancer and the expression was found to be associated with poor overall survival. Moreover, Jagged-1 expression is associated with a basal phenotype and recurrence in lymph node-negative breast cancer [42–44]. Very recently, it was found that Notch-1 and Notch-4 receptors could serve as prognostic markers in breast cancer [18]. Shi et al. [45] also found that the Notch family expression pattern in papillary bladder transitional cell carcinoma which was different from that in invasive bladder transitional cell carcinoma. Therefore, the expression of Notch-1 and Jagged-1 could potentially be a useful marker for survival of patients diagnosed with papillary bladder transitional cell carcinoma. More recently, it was reported that patients with cervical carcinomas positive for nuclear Notch-3 expression had significantly shorter overall survival than their peers whose tumors did not express nuclear Notch-3, suggesting that Notch-3 could be a prognostic marker in cervical carcinomas [46]. Further research toward exploration of the Notch signal as diagnostic and prognostic markers in human cancers requires in-depth investigations.

3. The role of Notch in drug resistance

Recently, Notch pathway has been reported to be involved in drug resistance. More importantly, the studies have demonstrated that Notch regulates the formation of cancer stem cells (CSCs) and contributes to the acquisition of the epithelial–mesenchymal transition (EMT) phenotype, which are critically associated with drug resistance [40,47]. Experimental evidence also revealed that Notch was involved in anti-cancer drug resistance, indicating that targeting Notch could be a novel therapeutic approach for the treatment of cancer by overcoming drug resistance of cancer cells, which may lead to the elimination of CSCs or EMT type cells which are typically drug-resistant, and are believed to be the "root cause" of tumor recurrence. Therefore, in the following sections, we have attempted to summarize the state of our knowledge on the functional role of Notch signaling pathway in drug resistance, and approaches by which one could overcome drug resistance for the successful treatment of most human malignancies.

3.1. Drug resistance

Chemotherapy is an important therapeutic strategy for cancer treatment and remains the mainstay for the management of human malignancies; however, chemotherapy fails to eliminate all tumor cells because of intrinsic or acquired drug resistance, which is the most common cause of tumor recurrence. Human cancers are generally initially responsive to standard chemotherapies; however, response is almost inevitably followed by the development of drug-resistant phenotype [48], which leads to tumor recurrence and metastasis. The mechanisms responsible for drug resistance are complex and still poorly understood. It may be due to either the specific nature and genetic background of the cancer cell itself or the genetic changes that follow toxic chemotherapy

[49]. Drug resistance to therapy is classified by two categories: intrinsic (*de novo*) and acquired. Intrinsic resistance would make the therapy ineffective because prior to receiving the therapy, the cancer cells have already resistant to anti-cancer drugs due to multiple mechanisms. Acquired resistance develops during the treatment, although the tumor cells were not initially resistant to anti-cancer drugs. The most common reasons for the acquisition of resistance to anti-cancer drugs are due to expression of one or more energy-dependent transporters that detect and eject anti-cancer drugs from cells, insensitivity to drug-induced apoptosis and the induction of drug-detoxification mechanisms [50]. For example, the ATP-binding cassette (ABC) drug transporters have been shown to protect tumor cells from chemotherapeutic agents. ABC transporters eject toxic drugs from cancer cells, leading to reducing the effect of drug's ability to kill the cancer cells. There are three ABC protein members that have been identified, which are ABCB1 (PGP, P-glycoprotein), ABCG2 (BCRP, breast cancer resistant protein), and ABCC1 (MRP1, multidrug resistance associated protein) [49,50].

Studies over the past years have shown that a number of genes are involved in chemotherapy drug resistance. These genes include K-ras, TOP1 (topoisomerase 1), ERCC1 (excision repair cross complementation 1), LRP (lung resistance-related protein), COX-2, cyclin D1, Bcl-2, Survivin, etc. [50–53]. Recently, many signaling pathways have been found to be involved in drug resistance such as PTEN, Akt, mTOR, NF-κB, EGFR, FGFR (fibroblast growth factor receptor), Raf/MEK/ERK, MAPK (mitogen-activated protein kinase), IGF (insulin-like growth factor), and Notch signaling pathway [54–61]. The main roles of these pathways (except Notch signaling pathway) in drug resistance have recently been reviewed [53–60]. Therefore, in this review article, we will focus our discussion on describing the role of Notch in drug resistance and summarize approaches by which one could overcome drug resistance.

3.2. Notch regulates EMT in drug resistance

Recent studies have shown that EMT is associated with drug resistance and cancer cell metastasis. It is now widely accepted that epithelial cells can acquire mesenchymal phenotype by a fundamental yet complex processes. The processes of EMT is a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype (mesenchymal phenotype) leading to increased motility and invasion [62]. During the acquisition of EMT characteristics, cells lose epithelial cell–cell junction, actin cytoskeleton reorganization and the expression of proteins that promote cell–cell contact such as E-cadherin and γ-catenin, and gains in the expression of mesenchymal markers such as vimentin, fibronectin, α-smooth muscle actin (SMA), and N-cadherin as well as increased activity of matrix metalloproteinases (MMPs) like MMP-2, MMP-3, and MMP-9, leading to an invasive phenotype [63]. Indeed, increasing evidence has shown the relationship between drug resistance and the existence of EMT phenotype. Fox instance, epithelial but not mesenchymal gene signature has been associated with sensitivity to the EGFR inhibitor erlotinib-mediated growth inhibition in lung cancer cells [64]. These results were confirmed in other types of tumors like head and neck squamous cell carcinoma and hepatocellular carcinoma as well as for the treatment of cancer with other EGFR inhibitors such as gefitinib and cetuximad [65,66]. The processes of EMT has also been shown to be important on conferring drug resistance characteristics to cancer cells against conventional therapeutics including taxol, vincristine, and oxaliplatin [67]. Consistent with these observations, recent studies has also shown the link between EMT and gemcitabine-resistant pancreatic cancer cells with increased invasive capacities, oxaliplatin-resistant colorectal cancer cells, lapatinib-resistant breast cancer, and paclitaxel-resistant ovarian carcinoma cells [68–71]. Therefore, the discovery of precise mechanisms that governs the acquisition of EMT phenotype in cancer cells would likely be useful for devising targeted therapeutic approaches in combination with conventional therapeutics for the treatment of human malignancies.

Notch signaling pathway has been reported to be involved with the acquisition of EMT in drug-resistant cancer cells. Our recently published data showed that pancreatic cancer cells that are gemcitabine-resistant (GR) have acquired EMT phenotype as evidenced by elongated fibroblastoid morphology, lower expression of epithelial marker E-cadherin, and higher expression of mesenchymal markers such as zinc-finger E-box binding homeobox 1 (ZEB1) and vimentin [70,72]. We also found that Notch-2 and Jagged-1 are highly up-regulated in GR cells. Moreover, down-regulation of Notch signaling by siRNA approach led to partial reversal of the EMT phenotype, resulting in the mesenchymal-to-epithelial transition (MET), which was associated with decreased expression of vimentin, ZEB1, Slug, Snail, and NF-κB [72]. These results provide molecular evidence indicating that the activation of Notch signaling is mechanistically linked with chemoresistance phenotype, which is consistent with the acquisition of EMT phenotype by pancreatic cancer cells, and further suggesting that the inactivation of Notch signaling by novel strategies could be a potential targeted therapeutic approach for overcoming chemoresistance toward the prevention of tumor progression and/or treatment of human cancer for which current conventional therapeutic strategies are highly disappointing.

3.3. Notch regulates cancer stem cell in drug resistance

Current cancer therapeutic strategies based on tumor regression may target and kill differentiated tumor cells, which constitute the bulk of the tumor, while sparing the rare cancer stem cell population. Cancer stem cells (CSCs) constitute a small subset of cancer cells that are a reservoir of self-sustaining cells with the exclusive ability to self-renew capacity leading to the maintenance of the tumor mass. The CSCs have been identified and isolated from tumors of the hematopoietic system, breast, lung, prostate, colon, brain, head and neck, and pancreas [73]. The CSCs are able to self-renew, differentiate, and regenerate to phenotypic cells of the original tumor when implanted into the severe combined immunodeficient mouse. Recently, CSCs have been believed to play critical roles in drug resistance and cancer metastasis especially because CSCs express drug transporters and DNA repair systems, which allow CSCs to resist the killing effects of the drug. For instance, ABC drug transporters have been shown to protect CSCs from chemotherapeutic agents [74,75]. Another mechanism is that CSCs accumulate mutations over time as a consequence of a long-term exposure to drug, which confer drug resistance phenotype acquired by the daughter cancer cells [76]. Thus, the molecular knowledge of drug resistance and metastasis with respect to CSCs in human cancer is considered very important, and the gain of such knowledge is likely to be helpful not only in the discovery of newer drugs but also in the design of novel therapeutic strategies for the treatment of human cancer with better treatment outcome.

Emerging evidence is clearly showing that Notch signaling plays critical roles in both stem cells and progenitor cells, suggesting that abnormal Notch signaling may contribute to carcinogenesis by deregulating the self-renewal of normal stem cells. For example, Phillips et al. [77] have reported that CSCs can be identified by phenotypic markers and their fate is controlled by the Notch pathway in breast cancer. Recombinant human erythropoietin receptor increased the numbers of stem cells and self-renewing capacity in a Notch-dependent fashion by induction of Jagged-1. Inhibitors of the Notch pathway blocked this effect, suggesting the mechanistic role of Notch signaling in the maintenance of cancer stem-like cell phenotype [77]. Farnie et al. [78] also provided evidence for breast cancer stem cells, and their studies have consistently shown that stem-like cells and breast cancer initiating populations can be enriched using cell surface markers CD44+/CD24− and, as such, these cells showed up-regulated genes including Notch that are known to contribute to cancer stem-like cells characteristics. It has also been reported that glioma stem cells have elevated

chemoresistance because of the high expression levels of drug-transporter proteins such as ABCG2. Furthermore, ABCG2 expression is also associated with proliferation, and the ABCG2-positive cells preferentially express several "stemness" genes such as Notch-1 [79]. Therefore, eradication of CSCs is an important goal for curing cancer, and thus the Notch pathway is considered an attractive target for treatment of cancer because targeting Notch will not only kill differentiated cancer cells but could also kill CSCs by overcoming drug resistance.

3.4. Notch cross talks with miRNAs in drug resistance

Recently, evidences suggest that microRNAs (miRNAs) play important roles in the regulation of drug resistance [80]. It is well known that the miRNAs elicit their regulatory effects in post-transcriptional regulation of genes by binding to the 3′ untranslated region (3′UTR) of target messenger RNA (mRNA). Either perfect or near perfect complimentary base pairing results in the degradation of the mRNA, while partial base pairing leads to translational inhibition to functional proteins [81]. Very interestingly, some miRNAs are thought to have oncogenic activity while others have tumor suppressor activity. Oncogenic miRNAs are up-regulated in cancer and contribute to its pathology through various mechanisms such as targeting tumor suppressor genes. In contrast to the oncogenic miRNAs, other miRNAs are considered to have tumor suppressor activity and are down-regulated in cancer [82]. Recent studies have suggested altered expression of specific miRNAs in drug-resistant tumor cells. For example, the expression of three miRNAs (miR-192, miR-424, and miR-98) was significantly up-regulated while the expression of three other miRNAs (miR-194, miR-200b, and miR-212) was down-regulated in docetaxel-resistant NSCLC cells [83]. Recently, Song et al. [84] found that the expression of miR-140 was associated with chemosensitivity to 5-fluorouracil (5-FU) and methotrexate in osteosarcoma. Specifically, blocking endogenous miR-140-sensitized resistant cancer cells to 5-FU treatment, whereas overexpression of miR-140 made tumor cells more resistant to 5-FU, suggesting that miR-140 could be a novel target to develop therapeutic strategy to overcome drug resistance. Increasing evidence clearly implicating the role of miRNAs in drug resistance, and in a recent review article, we have summarized the implication of miRNAs in drug resistance for designing novel cancer therapy [80]. Here, we will discuss further how miRNAs could cross talk with Notch pathway leading to drug resistance and how and what novel agents could be useful to overcome such a drug resistance phenotype of cancer cells.

One miRNA, namely miR-34, has been found to participate in Notch pathways regulation and has been reported to be involved drug resistance [85]. The miR-34 family is composed of three processed miRNAs: miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c share a common primary transcript [86]. The expression of miR-34a has been found to be lower or undetectable in pancreatic cancer, osteosarcoma, breast cancer, and non-small cell lung cancer, suggesting that miR-34a could function as a tumor suppressor gene [86]. Recently, Li et al. [87] reported that transfection of miR-34a to glioma cells down-regulated the protein expression of Notch-1, Notch-2, and CDK6. More recently, Ji et al. [88] reported that human gastric cancer cells with miR-34 restoration reduced the expression of target gene Notch. In parallel, the same group reported that Notch-1 and Notch-2 are downstream genes of miR-34 in pancreatic cancer cells because restoration of miR-34 expression in the pancreatic cancer cells down-regulated the expression of Notch-1 and Notch-2. Moreover, they reported that pancreatic cancer stem cells are enriched with tumor-initiating cells or CSCs with high levels of Notch-1/2 and loss of miR-34 [89], suggesting that miR-34 may be involved in pancreatic cancer stem cell self-renewal mediated by Notch signaling. More recently, Fujita et al. [85] demonstrated that miR-34a is down-regulated in drug-resistant prostate cancer cells, and ectopic overexpression of miR-34a resulted in growth inhibition

and attenuated chemoresistance to the anti-cancer drug camptothecin. Very recently, another study determined that miR-34a was down-regulated in doxorubicin and verapamil resistance MCF-7 breast cancer cells [90]. Collectively, these reports clearly suggest the role of miR-34 in drug resistance, which is in part mediated through the regulation of Notch signaling; however, further in-depth research is needed in order to fully understanding how miR-34 regulates the Notch pathway in drug-resistant cells and finding novel avenues by which one could up-regulate miR-34 would be highly innovative for designing novel treatment strategies for eliminating tumor cells that are the root cause of tumor recurrence and metastasis.

Another miRNA, miR-1, was markedly reduced in primary human hepatocellular carcinoma, prostate cancer, head and neck, and lung cancer [91,92]. Recently, miR-1 was also found to alter sensitivity of cancer cells to therapeutic agents. Nasser et al. [92] reported that ectopic miR-1 expression sensitize lung cancer cells to anti-cancer drug doxorubicin, suggesting that up-regulation of miR-1 has potential as a target for therapy against lung cancers. It has been reported that Notch ligand Dll-1 protein levels are negatively regulated by miR-1 [93]. In parallel, miR-1 directly targets the Notch ligand delta for repression [94], suggesting that miR-1 may regulate drug resistance in part via regulating the Notch signaling pathway. Recently, the alteration of miR-200 family was also found in drug-resistant cells. The miR-200 family has five members: miR-200a, miR-200b, miR-200c, miR-141, and miR-429. The expression of miR-200b was significantly down-regulated in docetaxel-resistant NSCLC cells [83]. Recently, many studies have shown that the miR-200 family regulates EMT which is associated with drug resistance. One study discovered that miR-200 expression regulates EMT in bladder cancer cells and reverses resistance to EGFR therapy [95]. Another recent study reported that miR-200c restored microtubule-binding chemotherapeutic agents in breast and ovarian cancer cells [96]. We also found that miR-200a, miR-200b, and miR-200c were down-regulated in gemcitabine-resistant pancreatic cancer cells, which show the acquisition of EMT phenotype. Furthermore, we have shown that re-expression of miR-200 family resulted in the down-regulation of ZEB1, slug, E-cadherin, and vimentin and increased cell sensitivity to gemcitabine [97]. In addition, we found that Notch-1 could be one of miR-200b targets because overexpression of miR-200 family significantly inhibited Notch-1 expression in gemcitabine-resistant pancreatic cancer cells and prostate cancer cells (unpublished data), suggesting that re-expression of miR-200 could increase drug sensitivity, which indeed could be mediated through the regulation of Notch signaling pathway. Thus, it is our belief that more and more miRNAs will be discovered, whose re-expression will make drug-resistant cells drug-sensitive, and such strategy could be useful in eliminating cancer cells with propensity of recurrence and metastasis.

3.5. Notch pathway in specific chemoresistance

Chemotherapy is critically important for cancer therapy; however, chemotherapy fails to eliminate all tumor cells due to chemoresistance either the de novo or acquired chemoresistance. Currently, chemoresistance is still the most common cause of tumor progression. Many cellular pathways have been found to be involved in drug resistance. Recent studies have demonstrated that Notch pathway plays a critical role in anti-cancer drug resistance as documented in the previous paragraphs. Here, we will further discuss the roles of Notch pathway in chemoresistance and a comprehensive list of Notch pathway that is involved in chemoresistance is presented in Table 1.

3.5.1. The role of Notch in anti-cisplatin resistance

Cisplatin is the most important chemotherapeutic agent for the treatment of human carcinoma including lung, ovarian, bladder, and testicular cancers. However, acquired resistance to cisplatin therapy is still a critical problem in the clinical management of cancer patients. Recent studies have shown that Notch may play a role in the mechanisms

Table 1
A comprehensive list of Notch pathway involved in chemoresistance.

Drug	Targeted genes	Cell or tissue	Reference
Cisplatin	Notch-1 was highly expressed in cisplatin resistance cells	Head and neck squamous cell, colorectal, and ovarian cancer cells	[98–101]
Doxorubicin	Notch-3 was up-regulated and contributed to the anti-cancer drug doxorubicin resistance through regulating p53 expression and DNA damage. GSI improved the cytotoxicity of the doxorubicin.	Hepatocellular carcinoma cells, myeloma cells	[114,116]
Erlotinib	GSI enhance the EGFR tyrosine kinase inhibitor erlotinib anti-tumor activity	Lung cancer	[112]
5-fluorouracil	5-FU induced NICD protein and activated Hes-1	Colon cancer cells	[108]
Gemcitabine	Inhibition of Notch-3 enhances sensitivity to gemcitabine. Notch-2 and Jagged-1 are highly up-regulated in gemcitabine resistance.	Pancreatic cancer cells	[72,102]
Gefitinib	Overexpression of Notch-1 contributes to the gefitinib resistance	Breast cancer cells	[111,113]
Melphalan	GSI improved the cytotoxicity of the melphalan	Myeloma cells	[116]
Mitoxantrone	Activation of Notch-1 resulted in the protection f rom mitoxantrone-induced apoptosis	Myeloma cells and malignant lymphoid cell lines	[115]
Oxaliplatin	Oxaliplatin induced NICD protein and activated Hes-1 GSI-sensitized cells to oxaliplatin	Colon cancer cells	[108]
Paclitaxel	Taxol enhanced the expression of Notch downstream gene CBF1. GSIs are useful for the chemotherapeutic treatment of taxol-resistant cancer cells.	Erythroleukemia, cervical, colorectal, ovarian cancer cells	[105–107]
Tamoxifen	Down-regulation of Notch-1 or GSI potentiated the effects of tamoxifen	Breast cancer cells	[27]
Taxol	GSIs enhance taxol-induced mitotic arrest and apoptosis of colon cancer cells	Erythroleukemia cells, cervical, colon, ovarian cancer cells	[105–107]
Taxetere	Down-regulation of Notch-1 signaling increased chemosensitivity to taxotere	Breast and prostate cancer cells	[104]
Trastuzumab	Notch-1 signaling regulates ErbB-2 transcription. Down-regulation of Notch-1 increased efficacy of trastuzumab and restored sensitivity in resistant cells	Breast cancer cells	[61,109,110]

of cisplatin resistance. One such study by Zhang et al. [98] demonstrated that the positive rate of Notch-1 was significantly higher in head and neck squamous cell carcinoma (HNSCC) than in normal squamous epithelium, and it was negatively correlated with cisplatin sensitivity. Moreover, Notch-1 was highly expressed in cisplatin-resistant HNSCC patients [99]. Further, cisplatin resistance of HNSCC was decreased after inhibition of Notch signaling [99]. In addition, combination of GSI and cisplatin elicits a striking induction of colorectal cancer cell death [100]. Human ovarian cancer-initiating cells enhanced chemoresistance to cisplatin and up-regulation of Notch-1 compared with parental tumor cells [101]. These results support the notion that inactivation of Notch pathway could be a novel strategy for patients who is likely respond to such chemotherapy.

3.5.2. The role of Notch in anti-gemcitabine resistance

Gemcitabine monotherapy (2′,2′-difluorodeoxycytidine), a deoxy-cytidine analogue, or its combination with other agents has become standard chemotherapy for the treatment of advanced human cancers. However, the effect of gemcitabine on survival has been disappointing, which could be due to many factors including intrinsic drug resistance or acquired drug resistance. For example, gemcitabine showed only about 5% partial response rate and imparts a progression-free survival interval ranging from 0.9 to 4.2 months in pancreatic cancer. This disappointing outcome strongly suggests that a better understanding of the mechanism by which chemoresistance arises is likely to lead to novel therapeutic strategies for the successful treatment of cancer patients. Recently, Notch signaling pathway was found to play a critical role in gemcitabine-resistant cancer cells. Yao et al. [102] demonstrated that Notch-3 siRNA suppressed Notch-3 expression and increased gemcitabine-induced, caspase-mediated apoptosis in pancreatic cancer. Moreover, inhibition of Notch-3 enhances sensitivity to gemcitabine in pancreatic cancer through an inactivation of PI3K/Akt-dependent pathway. We also found that Notch-2 and Jagged-1 are highly up-regulated in gemcitabine-resistant pancreatic cancer cells. Moreover,

down-regulation of Notch signaling by siRNA approach led to partial reversal of the EMT phenotype, which was associated with increased gemcitabine sensitivity [72].

3.5.3. The role of Notch in anti-taxotere resistance

Taxotere (Docetaxel), a member of the taxane family, has shown high efficacy in the treatment of a wide spectrum of solid tumors including prostate, breast, and gastric cancer [103]. It has been found that taxotere inhibits cell growth and induces apoptosis with down-regulation of some genes for cell proliferation, transcription factors, and oncogenesis and up-regulation of some genes related to the induction of apoptosis and cell cycle arrest in tumor cells, suggesting pleiotropic effects of taxotere on tumor cells. Clinical trials have shown that the combination chemotherapy using taxotere with other agents improves survival in cancer patients [103]. However, the effect of taxotere is also disappointed due to drug resistance. Recently, we found that taxotere-resistant DU145 prostate cells have high expression of Notch-1 (unpublished data), suggesting that Notch pathway is involved in taxotere resistance. Another group also reported that down-regulation of Notch-1 signaling increased chemosensitivity to taxotere and doxorubicin in breast cancer [104], indicating that Notch signaling may be a promising target for overcoming taxotere-resistant in breast cancer and other cancers.

3.5.4. The role of Notch in anti-taxol resistance

Taxol (Paclitaxel) is another anti-cancer chemotherapy drug. It is used for the treatment of breast, ovarian, lung, bladder, prostate, melanoma, esophageal, and other types of solid tumors. It has been reported that taxol could enhance the expression of Notch downstream gene CBF1 in erythroleukemia K562 cells and cervical carcinoma HeLa cells [105]. Recently, it was found that GSIs could enhance taxol-induced mitotic arrest and apoptosis of colon cancer cells both *in vitro* and *in vivo*, suggesting that GSIs could be useful for the treatment of taxol-resistant

colorectal cancers [106]. More recently, Mine et al. [107] reported that targeting Notch-1 was significant for novel treatments to eliminate taxol-resistant ovarian cancer stem cells. These limited emerging evidences suggests that overcoming taxol resistance could be achieved by inactivation of Notch signaling which would become a rational approach for the treatment of most human malignancies.

3.5.5. The role of Notch in anti-tamoxifen resistance

Tamoxifen is a well-known anti-cancer drug for the treatment of breast cancer. Certain types of breast cancer require estrogen to grow and tamoxifen blocks the actions of estrogen. Tamoxifen works by blocking the effect of estrogen, resulting in inhibiting gene transcription and tumor growth that are activated by estrogen. Resistance to tamoxifen is often seen in tumor cells that become estrogen independent; thus, tamoxifen cannot inhibit tumor growth. In a recent study, Rizzo et al. [27] found that down-regulation of Notch-1 by siRNA or GSI potentiated the effects of tamoxifen in breast cancer cells. Moreover, GSI in combination with tamoxifen caused regression of breast cancer cell growth in mice. These data indicate that the combinations of tamoxifen and Notch inhibitors may be effective in ERα (+) breast cancer, and such a combination treatment could eliminate the emergence of Tamoxifen resistance, which certainly would improve the treatment outcome of patients diagnosed with breast cancer.

3.5.6. The role of Notch in anti-oxaliplatin resistance

Oxaliplatin is a platinum-compound chemotherapy drug that acts as an alkylating agent. Oxaliplatin is used to treat colorectal cancer, and it is often given in combination with other anti-cancer drugs (5-fluorouracil and leucovorin). It has been shown that Notch-1 is up-regulated in colon cancer. Further, oxaliplatin or 5-FU could induce NICD protein and activated Hes-1 though an increase in the activity and expression of gamma-secretase complex. Therefore, GSI could sensitize cells to chemotherapy, which has been demonstrated showing synergistic activity with oxaliplatin and 5-FU [108]. The authors have summarized that colon cancer cells with up-regulated expression of Notch-1 could function as a protective mechanism in response to chemotherapy [108], further suggesting that combining GSIs with chemotherapy may be a novel strategy for overcoming chemoresistance in colon cancer.

3.5.7. The role of Notch in anti-trastuzumab resistance

Trastuzumab is the humanized, monoclonal antibody that directed against ErbB-2. It has shown efficacy causing improved overall survival for breast cancer patients. However, resistance to trastuzumab remains a major concern, specifically in women with metastatic breast cancer. It has been found that Notch-1 could contribute to trastuzumab resistance in breast cancer [61]. Notch-1 signaling regulates ErbB-2 transcription in ErbB-2-overexpressing breast carcinoma tumor-initiating cells, therefore affecting their self-renewal properties [109]. Trastuzumab increased the Notch-1 activity and its target gene expression. The expression of Notch-1, Hey-1, and Hes-5 was highly expressed in trastuzumab-resistant BT474 compared to trastuzumab-sensitive BT474 [110]. Moreover, down-regulation of Notch-1 increased efficacy of trastuzumab in BT474-sensitive cells and restored sensitivity in resistant cells. Furthermore, the growth of both trastuzumab-sensitive and -resistant cells was completely inhibited by combining trastuzumab plus Notch-1 siRNA. The Notch-1 siRNA or a GSI re-sensitized trastuzumab-resistant BT474 cells to trastuzumab [110], suggesting that Notch-1 might play a novel role in resistance to trastuzumab, which could be prevented or reversed by inhibiting Notch-1.

3.5.8. The role of Notch in other chemoresistance drugs

Notch signaling pathway was also found in many other chemoresistant cancer cells [111–113]. For example, Notch-3 was up-regulated and contributed to the anti-cancer drug doxorubicin resistance through regulating p53 expression and DNA damage in human hepatocellular

carcinoma (HCC) cell lines, suggesting that Notch-3 silencing in combination with chemotherapeutics could conceivably provide a novel strategy for HCC treatment [114]. Another study determined that Notch-1 signaling was involved in bone marrow stroma-mediated de novo melphalan and mitoxantrone resistance of myeloma [115]. Moreover, GSI significantly improved the cytotoxicity of the chemotherapeutic drugs doxorubicin and melphalan in myeloma cells, demonstrating that inhibition of Notch signaling prevents bone marrow-mediated drug resistance and sensitizes to chemotherapy [116]. There are currently more and more studies being done to uncover the resistance mechanism by Notch signaling pathway.

4. Targeting Notch to increase drug sensitivity

Notch signaling has been reported to be involved in drug resistance as documented in the previous paragraphs. Therefore, targeting Notch pathway for cancer therapy is a novel strategy for optimizing treatment outcome of conventional chemotherapy. Strategies to regulate Notch expression in cancers could be at many different levels. It is possible to interfere with Notch–ligand interactions, receptor activation, mono-ubiquitination, and NICD nuclear complex formation and inhibition of its translocation to the nuclear compartment (Fig. 1B). Notch signaling is activated via the activity of γ-secretase which became a target in cancer therapy. Several forms of γ-secretase inhibitors (GSIs) have been tested for anti-tumor effects. The GSI inhibits cell growth and could induce apoptosis in many human cancer cells, such as hepatoma cells, breast cancer cells, pancreatic cancer cells, and myeloma cells [1,7]. Recently, it was found that inhibition of Notch signaling with GSI-sensitized cells to chemotherapy and was synergistic with oxaliplatin and 5-FU, suggesting that combining GSI with chemotherapy may represent a novel approach for treating metastatic colon cancers as indicated above [108]. Recently, Gu et al. [99] reported that cisplatin resistance of HNSCC was decreased by inhibition of Notch signaling, suggesting that inactivation of Notch-1 could help HNSCC response to chemotherapy. Very recently, Song et al. [28] evaluated the effects of Notch-1 silencing on cisplatin-induced cytotoxicity in CaSki cervical cancer cells. They found that Notch-1 knockdown by siRNA significantly potentiated cisplatin-induced cytotoxicity, lowering the IC50 value of cisplatin in CaSki cells by almost two orders of magnitude. Collectively, all the published data suggest that targeting Notch pathway could increase drug sensitivity in human cancers; however, one of the major challenges is to eliminate unwanted toxicity associated with the GSI, especially the cytotoxicity in the gastrointestinal tract. Shih et al. [39] reported the possible mechanisms underlying the unwanted cytotoxicity of GSI. Notch signaling pathway is known to widely participate in cellular physiology in normal tissues; therefore, it is plausible that inactivation of γ-secretase may lead to the dysfunction of vital organs. Moreover, GSI do not exclusively target the Notch signaling pathways because γ-secretase has many substrates in addition to Notch receptors, such as several Notch ligands, v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ErbB4), CD44, etc. Further, GSI may target proteases other than γ-secretase. Therefore, GSI may have widespread adverse effects in vivo because proteases participate in a wide array of cellular functions [39].

In order to overcome such limitations, recent studies have shown that "natural agents", which are typically non-toxic to humans, including isoflavone, resveratrol, curcumin, withaferin-A, and others, could inhibit the Notch-1 expression. The studies from our laboratory have shown that genistein and curcumin down-regulated the transcription and translation of Notch-1 and its downstream genes, Hes-1, cyclin D1, Bcl-X$_L$, and NF-κB. Overexpression of Notch-1 by Notch-1 cDNA transfection abrogated genistein- and curcumin-induced apoptosis to a certain degree. Therefore, we strongly believe that down-regulation of Notch-1 by genistein and curcumin is mechanistically linked to cell proliferation and apoptotic processes [9,117–119]. In addition, studies from other laboratories have shown that resveratrol could induce apoptosis by inhibiting the Notch pathway mediated by p53 and PI3K/Akt in T-ALL [120]. Moreover, one

Chinese herb anti-tumor B was also found to inhibit Notch expression in a mouse lung tumor model [121]. Recently, it was reported that withaferin-A could inhibit Notch-1 signaling and thereby down-regulates pro-survival pathways, such as Akt/NF-κB/Bcl-2, in colon cancer cells [122]. Furthermore, recent studies have shown that natural agents could alter the expression of specific miRNAs that could regulate Notch signaling pathway. We found that re-expression of miR-200 by pre-miR-200 transfection or treatment of GR pancreatic cancer cells with isoflavone resulted in the up-regulation of miR-200, leading to increased sensitivity of GR cells to gemcitabine. Isoflavone also induced the expression of let-7, which could be linked to the treatment effects [97]. Considering the relatively non-toxic nature of natural agents, targeting the Notch pathway by these natural agents combined with conventional chemotherapy could be a novel and safer approach for achieving better treatment outcome; however, further in-depth preclinical and clinical studies are warranted in order to appreciate the value of natural agents in overcoming drug resistance to eliminate cancer cells that are the root cause of tumor recurrence and metastasis.

5. Conclusion and overall perspectives

In this review article, we attempted to summarize the role of Notch pathway in drug resistance; however, we could not cite all the published studies, and thus we sincerely apologize to those whose work has not been cited here due to space limitations. In conclusion, recent studies demonstrate that Notch signaling pathway may play critical roles in the regulation of anti-cancer drug sensitivity and resistance. Since Notch signaling pathway has been found to be involved in EMT and CSCs and deregulated expression of miRNAs, suggesting that up-regulation and down-regulation of specific miRNA that are intimately associated with Notch signaling could become a novel approach for overcoming drug resistance (Fig. 2). As such, high expression of Notch pathway can reduce response to anti-cancer agents such as cisplatin, doxorubicin, 5-fluorouracil, gemcitabine, tamoxifen, etc., and thus, down-regulation of Notch signaling by multiple approaches appears to be a novel strategy for increasing drug sensitivity of cancer cells to conventional chemotherapeutics. To that end, natural agents such as genistein, curcumin, resveratrol, and others could be very useful for the inhibition of Notch signaling pathway, which could lead to the inhibition of cancer growth, induction of apoptosis, reversal of EMT phenotype, elimination of drug-resistant CSCs, and thereby increasing drug sensitivity, which would be useful for treatment of cancer patients with better treatment outcome. In summary, our findings together with those reported in the literature are becoming an exciting area for further in-depth research toward targeted inactivation of Notch signaling proteins, especially by natural agents, as a novel therapeutic approach for increasing the drug sensitivity, and thereby improving the treatment outcome of cancer patients, which is believed to be due to eliminating the cancer cells that are the root cause of tumor recurrence and metastasis.

References

[1] L. Miele, Notch signaling, Clin. Cancer Res. 12 (2006) 1074–1079.
[2] L. Miele, B. Osborne, Arbiter of differentiation and death: Notch signaling meets apoptosis, J. Cell. Physiol. 181 (1999) 393–409.
[3] G. Weinmaster, The ins and outs of notch signaling, Mol. Cell Neurosci. 9 (1997) 91–102.
[4] R. Kopan, M.X. Ilagan, The canonical Notch signaling pathway: unfolding the activation mechanism, Cell 137 (2009) 216–233.
[5] M.E. Fortini, Notch signaling: the core pathway and its posttranslational regulation, Dev. Cell 16 (2009) 633–647.
[6] L. Miele, H. Miao, B.J. Nickoloff, Notch signaling as a novel cancer therapeutic target, Curr. Cancer Drug Targets 6 (2006) 313–323.
[7] P. Rizzo, C. Osipo, K. Foreman, T. Golde, B. Osborne, L. Miele, Rational targeting of Notch signaling in cancer, Oncogene 27 (2008) 5124–5131.
[8] Z. Wang, S. Banerjee, Y. Li, K.M. Rahman, Y. Zhang, F.H. Sarkar, Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{kappa}B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells, Cancer Res. 66 (2006) 2778–2784.
[9] Z. Wang, Y. Zhang, Y. Li, S. Banerjee, J. Liao, F.H. Sarkar, Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells, Mol. Cancer Ther. 5 (2006) 483–493.
[10] Z. Wang, Y. Li, S. Banerjee, F.H. Sarkar, Exploitation of the Notch signaling pathway as a novel target for cancer therapy, Anticancer Res. 28 (2008) 3621–3630.
[11] A.P. Weng, A. Lau, Notch signaling in T-cell acute lymphoblastic leukemia, Future Oncol. 1 (2005) 511–519.
[12] J. Sjolund, C. Manetopoulos, M.T. Stockhausen, H. Axelson, The Notch pathway in cancer: differentiation gone awry, Eur. J. Cancer 41 (2005) 2620–2629.
[13] J.L. Li, A.L. Harris, Notch signaling from tumor cells: a new mechanism of angiogenesis, Cancer Cell 8 (2005) 1–3.
[14] S.J. Bray, Notch signalling: a simple pathway becomes complex, Nat. Rev. Mol. Cell Biol. 7 (2006) 678–689.
[15] P.J. Real, A.A. Ferrando, Notch inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia, Leukemia 23 (2009) 1374–1377.
[16] L. Qiao, B.C. Wong, Role of Notch signaling in colorectal cancer, Carcinogenesis 30 (2009) 1979–1986.
[17] A.R. Sandy, I. Maillard, Notch signaling in the hematopoietic system, Expert Opin. Biol. Ther. 9 (2009) 1383–1398.
[18] K. Yao, P. Rizzo, P. Rajan, K. Albain, K. Rychlik, S. Sha, L. Miele, Notch-1 and Notch-4 receptors as prognostic markers in breast cancer, Int. J. Surg. Pathol. (2010) (May 5, Electronic publication ahead of print).
[19] A.M. Jubb, E.J. Soilleux, H. Turley, G. Steers, A. Parker, I. Low, J. Blades, J.L. Li, P. Allen, R. Leek, I. Noguera-Troise, K.C. Gatter, G. Thurston, A.L. Harris, Expression of vascular notch ligand delta-like 4 and inflammatory markers in breast cancer, Am. J. Pathol. 176 (2010) 2019–2028.
[20] M. Wang, L. Xue, Q. Cao, Y. Lin, Y. Ding, P. Yang, L. Che, Expression of Notch1, Jagged1 and beta-catenin and their clinicopathological significance in hepatocellular carcinoma, Neoplasma 56 (2009) 533–541.
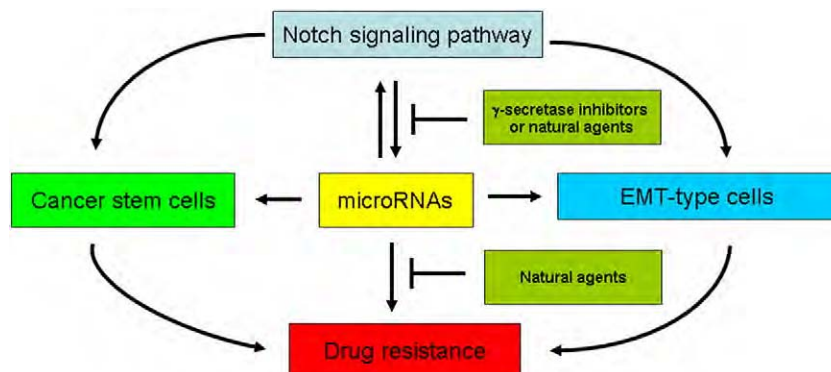
Fig. 2. The role of Notch signaling pathway in the progression of cancer, and during the acquisition of EMT phenotype, and the formation of cancer stem cells, leading to drug resistance. Natural agents and γ-secretase inhibitors could be useful for targeting Notch signaling pathway proteins, which could enhance the sensitivity of chemotherapeutic drugs.

[21] J. Gao, Y. Chen, K.C. Wu, J. Liu, Y.Q. Zhao, Y.L. Pan, R. Du, G.R. Zheng, Y.M. Xiong, H.L. Xu, D.M. Fan, RUNX3 directly interacts with intracellular domain of Notch1 and suppresses Notch signaling in hepatocellular carcinoma cells, Exp. Cell Res. 316 (2010) 149–157.

[22] C. Wang, R. Qi, N. Li, Z. Wang, H. An, Q. Zhang, Y. Yu, X. Cao, Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human hepatocellular carcinoma cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression, J. Biol. Chem. 284 (2009) 16183–16190.

[23] G.P. Dotto, Notch tumor suppressor function, Oncogene 27 (2008) 5115–5123.

[24] M. Nicolas, A. Wolfer, K. Raj, J.A. Kummer, P. Mill, N.M. van, C.C. Hui, H. Clevers, G.P. Dotto, F. Radtke, Notch1 functions as a tumor suppressor in mouse skin, Nat. Genet. 33 (2003) 416–421.

[25] S. Demehri, A. Turkoz, R. Kopan, Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment, Cancer Cell 16 (2009) 55–66.

[26] J. Panelos, D. Massi, Emerging role of Notch signaling in epidermal differentiation and skin cancer, Cancer Biol. Ther. 8 (2009) 1986–1993.

[27] P. Rizzo, H. Miao, G. D'Souza, C. Osipo, L.L. Song, J. Yun, H. Zhao, J. Mascarenhas, D. Wyatt, G. Antico, L. Hao, K. Yao, P. Rajan, C. Hicks, K. Siziopikou, S. Selvaggi, A. Bashir, D. Bhandari, A. Marchese, U. Lendahl, J.Z. Qin, D.A. Tonetti, K. Albain, B.J. Nickoloff, L. Miele, Cross-talk between notch and the estrogen receptor in breast cancer suggests novel therapeutic approaches, Cancer Res. 68 (2008) 5226–5235.

[28] L.L. Song, Y. Peng, J. Yun, P. Rizzo, V. Chaturvedi, S. Weijzen, W.M. Kast, P.J. Stone, L. Santos, A. Loredo, U. Lendahl, G. Sonenshein, B. Osborne, J.Z. Qin, A. Pannuti, B.J. Nickoloff, L. Miele, Notch-1 associates with IKKalpha and regulates IKK activity in cervical cancer cells, Oncogene 27 (2008) 5833–5844.

[29] Z. Wang, Y. Li, S. Banerjee, D. Kong, A. Ahmad, V. Nogueira, N. Hay, F.H. Sarkar, Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-kappaB signaling pathways, J. Cell. Biochem. 109 (2010) 726–736.

[30] Y. Zhang, Z. Wang, F. Ahmed, S. Banerjee, Y. Li, F.H. Sarkar, Down-regulation of Jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells, Int. J. Cancer 119 (2006) 2071–2077.

[31] O.J. De La, L.C. Murtaugh, Notch and Kras in pancreatic cancer: at the crossroads of mutation, differentiation and signaling, Cell Cycle 8 (2009) 1860–1864.

[32] C. Osipo, T.E. Golde, B.A. Osborne, L.A. Miele, Off the beaten path: the complex cross talk between Notch and NF-kappaB, Lab. Invest. 88 (2008) 11–17.

[33] M.V. Sundaram, The love–hate relationship between Ras and Notch, Genes Dev. 19 (2005) 1825–1839.

[34] Z. Wang, R. Sengupta, S. Banerjee, Y. Li, Y. Zhang, K.M. Rahman, A. Aboukameel, R. Mohammad, A.P. Majumdar, J.L. Abbruzzese, F.H. Sarkar, Epidermal growth factor receptor-related protein inhibits cell growth and invasion in pancreatic cancer, Cancer Res. 66 (2006) 7653–7660.

[35] Z. Wang, D. Kong, S. Banerjee, Y. Li, N.V. Adsay, J. Abbruzzese, F.H. Sarkar, Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling, Cancer Res. 67 (2007) 11377–11385.

[36] S. Weijzen, P. Rizzo, M. Braid, R. Vaishnav, S.M. Jonkheer, A. Zlobin, B.A. Osborne, S. Gottipati, J.C. Aster, W.C. Hahn, M. Rudolf, K. Siziopikou, W.M. Kast, L. Miele, Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells, Nat. Med. 8 (2002) 979–986.

[37] O.J. De La, L.C. Murtaugh, Notch signaling: where pancreatic cancer and differentiation meet? Gastroenterology 136 (2009) 1499–1502.

[38] U. Koch, F. Radtke, Notch and cancer: a double-edged sword, Cell. Mol. Life Sci. 64 (2007) 2746–2762.

[39] I. Shih, T.L. Wang, Notch signaling, gamma-secretase inhibitors, and cancer therapy, Cancer Res. 67 (2007) 1879–1882.

[40] Z. Wang, Y. Li, D. Kong, A. Ahmad, S. Banerjee, F.H. Sarkar, Cross-talk between miRNA and Notch signaling pathways in tumor development and progression, Cancer Lett. 292 (2010) 141–148.

[41] S. Santagata, F. Demichelis, A. Riva, S. Varambally, M.D. Hofer, J.L. Kutok, R. Kim, J. Tang, J.E. Montie, A.M. Chinnaiyan, M.A. Rubin, J.C. Aster, JAGGED1 expression is associated with prostate cancer metastasis and recurrence, Cancer Res. 64 (2004) 6854–6857.

[42] M. Reedijk, S. Odorcic, L. Chang, H. Zhang, N. Miller, D.R. McCready, G. Lockwood, S.E. Egan, High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival, Cancer Res. 65 (2005) 8530–8537.

[43] M. Reedijk, D. Pinnaduwage, B.C. Dickson, A.M. Mulligan, H. Zhang, S.B. Bull, F.P. O'Malley, S.E. Egan, I.L. Andrulis, JAG1 expression is associated with a basal phenotype and recurrence in lymph node-negative breast cancer, Breast Cancer Res. Treat. 111 (2008) 439–448.

[44] B.C. Dickson, A.M. Mulligan, H. Zhang, G. Lockwood, F.P. O'Malley, S.E. Egan, M. Reedijk, High-level JAG1 mRNA and protein predict poor outcome in breast cancer, Mod. Pathol. 20 (2007) 685–693.

[45] T.P. Shi, H. Xu, J.F. Wei, X. Ai, X. Ma, B.J. Wang, Z.H. Ju, G.X. Zhang, C. Wang, Z.Q. Wu, X. Zhang, Association of low expression of notch-1 and jagged-1 in human papillary bladder cancer and shorter survival, J. Urol. 180 (2008) 361–366.

[46] S. Yeasmin, K. Nakayama, M.T. Rahman, M. Rahman, M. Ishikawa, K. Iida, Y. Ohtuski, H. Kobayashi, S. Nakayama, K. Miyazaki, Expression of nuclear Notch3 in cervical squamous cell carcinomas and its association with adverse clinical outcomes, Gynecol. Oncol. 117 (2010) 409–416.

[47] Z. Wang, Y. Li, S. Banerjee, F.H. Sarkar, Emerging role of Notch in stem cells and cancer, Cancer Lett. 279 (2009) 8–12.

[48] H.J. Broxterman, K.J. Gotink, H.M. Verheul, Understanding the causes of multidrug resistance in cancer: a comparison of doxorubicin and sunitinib, Drug Resist. Updat. 12 (2009) 114–126.

[49] G. Szakacs, J.K. Paterson, J.A. Ludwig, C. Booth-Genthe, M.M. Gottesman, Targeting multidrug resistance in cancer, Nat. Rev. Drug Discov. 5 (2006) 219–234.

[50] M.M. Gottesman, Mechanisms of cancer drug resistance, Annu. Rev. Med. 53 (2002) 615–627.

[51] A. Bardelli, S. Siena, Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer, J. Clin. Oncol. 28 (2010) 1254–1261.

[52] B. Liu, L. Qu, H. Tao, Cyclo-oxygenase 2 up-regulates the effect of multidrug resistance, Cell Biol. Int. 34 (2010) 21–25.

[53] A. Lopez-Chavez, C.A. Carter, G. Giaccone, The role of KRAS mutations in resistance to EGFR inhibition in the treatment of cancer, Curr. Opin. Investig. Drugs 10 (2009) 1305–1314.

[54] J. LoPiccolo, G.M. Blumenthal, W.B. Bernstein, P.A. Dennis, Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations, Drug Resist. Updat. 11 (2008) 32–50.

[55] A.W. Hendrickson, P. Haluska, Resistance pathways relevant to insulin-like growth factor-1 receptor-targeted therapy, Curr. Opin. Investig. Drugs 10 (2009) 1032–1040.

[56] S.A. Kono, M.E. Marshall, K.E. Ware, L.E. Heasley, The fibroblast growth factor receptor signaling pathway as a mediator of intrinsic resistance to EGFR-specific tyrosine kinase inhibitors in non-small cell lung cancer, Drug Resist. Updat. 12 (2009) 95–102.

[57] E.A. Hopper-Borge, R.E. Nasto, V. Ratushny, L.M. Weiner, E.A. Golemis, I. Astsaturov, Mechanisms of tumor resistance to EGFR-targeted therapies, Expert Opin. Ther. Targets 13 (2009) 339–362.

[58] K.K. Haagenson, G.S. Wu, The role of MAP kinases and MAP kinase phosphatase-1 in resistance to breast cancer treatment, Cancer Metastasis Rev. 29 (2010) 143–149.

[59] Y. Lin, L. Bai, W. Chen, S. Xu, The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy, Expert Opin. Ther. Targets 14 (2010) 45–55.

[60] B.H. Jiang, L.Z. Liu, Role of mTOR in anticancer drug resistance: perspectives for improved drug treatment, Drug Resist. Updat. 11 (2008) 63–76.

[61] K. Mehta, C. Osipo, Trastuzumab resistance: role for Notch signaling, Sci. World J. 9 (2009) 1438–1448.

[62] J.P. Thiery, H. Acloque, R.Y. Huang, M.A. Nieto, Epithelial–mesenchymal transitions in development and disease, Cell 139 (2009) 871–890.

[63] J.P. Thiery, J.P. Sleeman, Complex networks orchestrate epithelial–mesenchymal transitions, Nat. Rev. Mol. Cell Biol. 7 (2006) 131–142.

[64] R.L. Yauch, T. Januario, D.A. Eberhard, G. Cavet, W. Zhu, L. Fu, T.Q. Pham, R. Soriano, J. Stinson, S. Seshagiri, Z. Modrusan, C.Y. Lin, V. O'Neill, L.C. Amler, Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of erlotinib in lung cancer patients, Clin. Cancer Res. 11 (2005) 8686–8698.

[65] A. Voulgari, A. Pintzas, Epithelial–mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic, Biochim. Biophys. Acta 1796 (2009) 75–90.

[66] B.A. Frederick, B.A. Helfrich, C.D. Coldren, D. Zheng, D. Chan, P.A. Bunn Jr., D. Raben, Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma, Mol. Cancer Ther. 6 (2007) 1683–1691.

[67] M. Sabbah, S. Emami, G. Redeuilh, S. Julien, G. Prevost, A. Zimber, R. Ouelaa, M. Bracke, W.O. De, C. Gespach, Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers, Drug Resist. Updat. 11 (2008) 123–151.

[68] H. Kajiyama, K. Shibata, M. Terauchi, M. Yamashita, K. Ino, A. Nawa, F. Kikkawa, Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells, Int. J. Oncol. 31 (2007) 277–283.

[69] G.E. Konecny, N. Venkatesan, G. Yang, J. Dering, C. Ginther, R. Finn, M. Rahmeh, M.S. Fejzo, D. Toft, S.W. Jiang, D.J. Slamon, K.C. Podratz, Activity of lapatinib a novel HER2 and EGFR dual kinase inhibitor in human endometrial cancer cells, Br. J. Cancer 98 (2008) 1076–1084.

[70] A.N. Shah, J.M. Summy, J. Zhang, S.I. Park, N.U. Parikh, G.E. Gallick, Development and characterization of gemcitabine-resistant pancreatic tumor cells, Ann. Surg. Oncol. 14 (2007) 3629–3637.

[71] A.D. Yang, F. Fan, E.R. Camp, B.G.van, W. Liu, R. Somcio, M.J. Gray, H. Cheng, P.M. Hoff, L.M. Ellis, Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines, Clin. Cancer Res. 12 (2006) 4147–4153.

[72] Z. Wang, Y. Li, D. Kong, S. Banerjee, A. Ahmad, A.S. Azmi, S. Ali, J.L. Abbruzzese, G.E. Gallick, F.H. Sarkar, Acquisition of epithelial–mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway, Cancer Res. 69 (2009) 2400–2407.

[73] C. Tang, B.T. Ang, S. Pervaiz, Cancer stem cell: target for anti-cancer therapy, FASEB J. 21 (2007) 3777–3785.

[74] J. Styczynski, T. Drewa, Leukemic stem cells: from metabolic pathways and signaling to a new concept of drug resistance targeting, Acta Biochim. Pol. 54 (2007) 717–726.

[75] S. Zhou, J.D. Schuetz, K.D. Bunting, A.M. Colapietro, J. Sampath, J.J. Morris, I. Lagutina, G.C. Grosveld, M. Osawa, H. Nakauchi, B.P. Sorrentino, The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype, Nat. Med. 7 (2001) 1028–1034.

[76] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells, Nature 414 (2001) 105–111.

[77] T.M. Phillips, K. Kim, E. Vlashi, W.H. McBride, F. Pajonk, Effects of recombinant erythropoietin on breast cancer-initiating cells, Neoplasia 9 (2007) 1122–1129.

[78] G. Farnie, R.B. Clarke, Mammary stem cells and breast cancer—role of Notch signalling, Stem Cell Rev. 3 (2007) 169–175.

[79] L. Patrawala, T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool, D.G. Tang, Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and A, Cancer Res. 65 (2005) 6207–6219.

[80] F.H. Sarkar, Y. Li, Z. Wang, D. Kong, S. Ali, Implication of microRNAs in drug resistance for designing novel cancer therapy, Drug Resist. Updat. 13 (2010) 57–66.

[81] C.M. Croce, G.A. Calin, miRNAs, cancer, and stem cell division, Cell 122 (2005) 6–7.

[82] C.M. Croce, Causes and consequences of microRNA dysregulation in cancer, Nat. Rev. Genet. 10 (2009) 704–714.

[83] W. Rui, F. Bing, S. Hai-Zhu, D. Wei, C. Long-Bang, Identification of microRNA profiles in docetaxel-resistant human non-small cell lung carcinoma cells (SPC-A1), J. Cell. Mol. Med. 14 (2010) 206–214.

[84] B. Song, Y. Wang, Y. Xi, K. Kudo, S. Bruheim, G.I. Botchkina, E. Gavin, Y. Wan, A. Formentini, M. Kornmann, O. Fodstad, J. Ju, Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells, Oncogene 28 (2009) 4065–4074.

[85] Y. Fujita, K. Kojima, N. Hamada, R. Ohhashi, Y. Akao, Y. Nozawa, T. Deguchi, M. Ito, Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells, Biochem. Biophys. Res. Commun. 377 (2008) 114–119.

[86] H. Hermeking, The miR-34 family in cancer and apoptosis, Cell Death Differ. 17 (2010) 193–199.

[87] Y. Li, F. Guessous, Y. Zhang, C. Dipierro, B. Kefas, E. Johnson, L. Marcinkiewicz, J. Jiang, Y. Yang, T.D. Schmittgen, B. Lopes, D. Schiff, B. Purow, R. Abounader, MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes, Cancer Res. 69 (2009) 7569–7576.

[88] Q. Ji, X. Hao, Y. Meng, M. Zhang, J. Desano, D. Fan, L. Xu, Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres, BMC Cancer 8 (2008) 266.

[89] Q. Ji, X. Hao, M. Zhang, W. Tang, M. Yang, L. Li, D. Xiang, J.T. Desano, G.T. Bommer, D. Fan, E.R. Fearon, T.S. Lawrence, L. Xu, MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells, PLoS ONE 4 (2009) e6816.

[90] G.Q. Chen, Z.W. Zhao, H.Y. Zhou, Y.J. Liu, H.J. Yang, Systematic analysis of microRNA involved in resistance of the MCF-7 human breast cancer cell to doxorubicin, Med. Oncol. 27 (2010) 406–415.

[91] J. Datta, H. Kutay, M.W. Nasser, G.J. Nuovo, B. Wang, S. Majumder, C.G. Liu, S. Volinia, C.M. Croce, T.D. Schmittgen, K. Ghoshal, S.T. Jacob, Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis, Cancer Res. 68 (2008) 5049–5058.

[92] M.W. Nasser, J. Datta, G. Nuovo, H. Kutay, T. Motiwala, S. Majumder, B. Wang, S. Suster, S.T. Jacob, K. Ghoshal, Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1, J. Biol. Chem. 283 (2008) 33394–33405.

[93] K.N. Ivey, A. Muth, J. Arnold, F.W. King, R.F. Yeh, J.E. Fish, E.C. Hsiao, R.J. Schwartz, B. R. Conklin, H.S. Bernstein, D. Srivastava, MicroRNA regulation of cell lineages in mouse and human embryonic stem cells, Cell Stem Cell 2 (2008) 219–229.

[94] C. Kwon, Z. Han, E.N. Olson, D. Srivastava, MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling, Proc. Natl Acad. Sci. U. S. A. 102 (2005) 18986–18991.

[95] L. Adam, M. Zhong, W. Choi, W. Qi, M. Nicoloso, A. Arora, G. Calin, H. Wang, A. Siefker-Radtke, D. McConkey, M. Bar-Eli, C. Dinney, miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy, Clin. Cancer Res. 15 (2009) 5060–5072.

[96] D.R. Cochrane, N.S. Spoelstra, E.N. Howe, S.K. Nordeen, J.K. Richer, MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents, Mol. Cancer Ther. 8 (2009) 1055–1066.

[97] Y. Li, T.G. Vandenboom, D. Kong, Z. Wang, S. Ali, P.A. Philip, F.H. Sarkar, Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells, Cancer Res. 69 (2009) 6704–6712.

[98] Z.P. Zhang, Y.L. Sun, L. Fu, F. Gu, L. Zhang, X.S. Hao, Correlation of Notch1 expression and activation to cisplatin-sensitivity of head and neck squamous cell carcinoma, Ai Zheng 28 (2009) 100–103.

[99] F. Gu, Y. Ma, Z. Zhang, J. Zhao, H. Kobayashi, L. Zhang, L. Fu, Expression of Stat3 and Notch1 is associated with cisplatin resistance in head and neck squamous cell carcinoma, Oncol. Rep. 23 (2010) 671–676.

[100] T. Aleksic, S.M. Feller, Gamma-secretase inhibition combined with platinum compounds enhances cell death in a large subset of colorectal cancer cells, Cell Commun. Signal. 6 (2008) 8.

[101] S. Zhang, C. Balch, M.W. Chan, H.C. Lai, D. Matei, J.M. Schilder, P.S. Yan, T.H. Huang, K.P. Nephew, Identification and characterization of ovarian cancer-initiating cells from primary human tumors, Cancer Res. 68 (2008) 4311–4320.

[102] J. Yao, C. Qian, Inhibition of Notch3 enhances sensitivity to gemcitabine in pancreatic cancer through an inactivation of PI3K/Akt-dependent pathway, Med. Oncol. (2009) (Oct 9, Electronic publication ahead of print).

[103] V.E. Chiuri, N. Silvestris, V. Lorusso, A. Tinelli, Efficacy and safety of the combination of docetaxel (Taxotere) with targeted therapies in the treatment of solid malignancies, Curr. Drug Targets 10 (2009) 982–1000.

[104] S. Zang, F. Chen, J. Dai, D. Guo, W. Tse, X. Qu, D. Ma, C. Ji, RNAi-mediated knockdown of Notch-1 leads to cell growth inhibition and enhanced chemosensitivity in human breast cancer, Oncol. Rep. 23 (2010) 893–899.

[105] T.S. Yeh, R.H. Hsieh, S.C. Shen, S.H. Wang, M.J. Tseng, C.M. Shih, J.J. Lin, Nuclear betaII-tubulin associates with the activated notch receptor to modulate notch signaling, Cancer Res. 64 (2004) 8334–8340.

[106] T. Akiyoshi, M. Nakamura, K. Yanai, S. Nagai, J. Wada, K. Koga, H. Nakashima, N. Sato, M. Tanaka, M. Katano, Gamma-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells, Gastroenterology 134 (2008) 131–144.

[107] T. Mine, S. Matsueda, H. Gao, Y. Li, K.K. Wong, G.E. Peoples, S. Ferrone, C.G. Ioannides, Created Gli-1 duplex short-RNA (i-Gli-RNA) eliminates CD44 Hi progenitors of taxol-resistant ovarian cancer cells, Oncol. Rep. 23 (2010) 1537–1543.

[108] R.D. Meng, C.C. Shelton, Y.M. Li, L.X. Qin, D. Notterman, P.B. Paty, G.K. Schwartz, gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity, Cancer Res. 69 (2009) 573–582.

[109] A. Magnifico, L. Albano, S. Campaner, D. Delia, F. Castiglioni, P. Gasparini, G. Sozzi, E. Fontanella, S. Menard, E. Tagliabue, Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to trastuzumab, Clin. Cancer Res. 15 (2009) 2010–2021.

[110] C. Osipo, P. Patel, P. Rizzo, A.G. Clementz, L. Hao, T.E. Golde, L. Miele, ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor, Oncogene 27 (2008) 5019–5032.

[111] J. Dai, D. Ma, S. Zang, D. Guo, X. Qu, J. Ye, C. Ji, Cross-talk between Notch and EGFR signaling in human breast cancer cells, Cancer Invest. 27 (2009) 533–540.

[112] J. Konishi, K.S. Kawaguchi, H. Vo, N. Haruki, A. Gonzalez, D.P. Carbone, T.P. Dang, Gamma-secretase inhibitor prevents Notch3 activation and reduces proliferation in human lung cancers, Cancer Res. 67 (2007) 8051–8057.

[113] M.P. Piechocki, G.H. Yoo, S.K. Dibbley, F. Lonardo, Breast cancer expressing the activated HER2/neu is sensitive to gefitinib in vitro and in vivo and acquires resistance through a novel point mutation in the HER2/neu, Cancer Res. 67 (2007) 6825–6843.

[114] C. Giovannini, L. Gramantieri, P. Chieco, M. Minguzzi, F. Lago, S. Pianetti, E. Ramazzotti, K.B. Marcu, L. Bolondi, Selective ablation of Notch3 in HCC enhances doxorubicin's death promoting effect by a p53 dependent mechanism, J. Hepatol. 50 (2009) 969–979.

[115] Y. Nefedova, P. Cheng, M. Alsina, W.S. Dalton, D.I. Gabrilovich, Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines, Blood 103 (2004) 3503–3510.

[116] Y. Nefedova, D.M. Sullivan, S.C. Bolick, W.S. Dalton, D.I. Gabrilovich, Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy, Blood 111 (2008) 2220–2229.

[117] Z. Wang, Y. Zhang, S. Banerjee, Y. Li, F.H. Sarkar, Inhibition of nuclear factor kappab activity by genistein is mediated via Notch-1 signaling pathway in pancreatic cancer cells, Int. J. Cancer 118 (2006) 1930–1936.

[118] Z. Wang, Y. Zhang, S. Banerjee, Y. Li, F.H. Sarkar, Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells, Cancer 106 (2006) 2503–2513.

[119] Z. Wang, S. Desmoulin, S. Banerjee, D. Kong, Y. Li, R.L. Deraniyagala, J. Abbruzzese, F. H. Sarkar, Synergistic effects of multiple natural products in pancreatic cancer cells, Life Sci. 83 (2008) 293–300.

[120] V. Cecchinato, R. Chiaramonte, M. Nizzardo, B. Cristofaro, A. Basile, G.V. Sherbet, P. Comi, Resveratrol-induced apoptosis in human T-cell acute lymphoblastic leukaemia MOLT-4 cells, Biochem. Pharmacol. 74 (2007) 1568–1574.

[121] Z. Zhang, Y. Wang, R. Yao, J. Li, Y. Yan, R.M. La, W.L. Lemon, C.J. Grubbs, R.A. Lubet, M. You, Cancer chemopreventive activity of a mixture of Chinese herbs (antitumor B) in mouse lung tumor models, Oncogene 23 (2004) 3841–3850.

[122] S. Koduru, R. Kumar, S. Srinivasan, M.B. Evers, C. Damodaran, Notch-1 inhibition by Withaferin-A: a therapeutic target against colon carcinogenesis, Mol. Cancer Ther. 9 (2010) 202–210.

Mini-review

Cross-talk between miRNA and Notch signaling pathways in tumor development and progression

Zhiwei Wang, Yiwei Li, Dejuan Kong, Aamir Ahmad, Sanjeev Banerjee, Fazlul H. Sarkar *

Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, MI, United States

ARTICLE INFO

ABSTRACT

Notch signaling pathways are known to regulate many cellular processes, including cell proliferation, apoptosis, migration, invasion, and angiogenesis, and is one of the most important signaling pathway during normal development. Recently, emerging evidences suggest that microRNAs (miRNAs) can function as key regulators of various biological and pathologic processes during tumor development and progression. Notch signaling has also been reported to be regulated through cross-talk with many pathways and factors where miRNAs appears to play a major role. This article will provide a brief overview of the published evidences for the cross-talks between Notch and miRNAs. Further, we summarize how targeting miRNAs by natural agents could become a novel and safer approach for the prevention of tumor progression and treatment.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Notch signaling

In recent years, we have witnessed the sudden explosion in the literature regarding the role of Notch signaling in tumor progression. It has become clear that Notch signaling is involved in cell proliferation, survival, apoptosis, and differentiation which affects the development and function of many organs [1]. Notch genes encode proteins which can be activated by binding of a family of its ligands. The four members of Notch receptors have been identified to date in mammals, including Notch-1 to -4. Five Notch ligands have been found in mammals: Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-like 4), Jagged-1 and Jagged-2 [1]. Notch signaling is initiated by binding of the Notch transmembrane receptors with their specific ligands between two neighboring cells. Upon activation, Notch is cleaved, releasing the Notch intracellular domain (NICD)

through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase complex. The NICD can subsequently translocate into the nucleus for transcriptional activation of Notch target genes [2] (Fig. 1). Inhibiting γ-secretase function would prevent the cleavage of the Notch receptor, resulting in blocking the Notch signal transduction signaling [3]. Therefore, γ-secretase inhibitors could be useful for the treatment of human malignancies, which are being tested in clinical trials (see website: www.clinicaltrials.gov). In the absence of NICD, transcription of Notch target genes is inhibited by a repressor complex mediated by the CSL (C protein binding factor 1/Suppressor of Hairless/Lag-1). When NICD enters the nucleus, it binds to CSL and recruits transcription activators to the CSL complex, leading to convert it from a transcriptional repressor into a transcription activator complex [3]. A few Notch target genes have been identified, some of which are dependent on Notch signaling in multiple tissues, while others are tissue specific. Notch target genes include Hes (Hairy enhance of split) family, Hey (Hairy/enhancer of spit related with YRPW motif), nuclear factor-kappa B (NF-κB), vascular growth factor receptor (VEGF),

* Corresponding author. Address: Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, 9374 Scott Hall, 540 E Canfield, Detroit, MI 48201, United States. Tel.: +1 313 576 8327; fax: +1 313 576 8389.

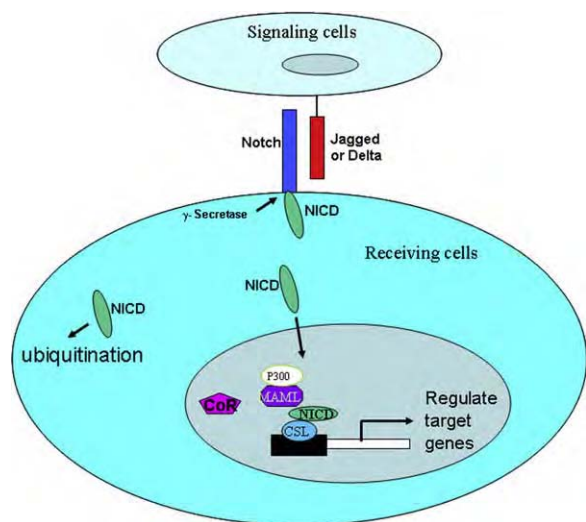E-mail address: fsarkar@med.wayne.edu (F.H. Sarkar).

Fig. 1. Schematic of Notch signaling. Notch signaling is initiated by binding of the Notch transmembrane receptors with their specific ligands between two neighboring cells. Upon activation, Notch is cleaved, releasing the Notch intracellular domain (NICD) through cleavage by γ-secretase complex. The NICD can subsequently translocate into the nucleus for transcriptional activation of Notch target genes. When NICD enters the nucleus, co-repressors associated with CSL (CBF1/Suppressor of Hairless/Lag-1) are displaced and a transcriptionally active complex consisting of CSL, NICD, Mastermind, and other co-activators is formed, which converts CSL from a transcriptional repressor into an activator, leading to activation of Notch target genes.

mammalian target of rapamycin (mTOR), cyclin D1, c-myc, p21, p27, Akt, etc. [4–7].

It has been well documented that Notch signaling maintains the balance between cell proliferation, differentiation and apoptosis. Therefore, alterations in Notch signaling are considered to be associated with tumorigenesis. Indeed, it has been reported that Notch genes are abnormally regulated in many human malignancies [8–10]. These observations suggest that dysfunction of NICD prevents differentiation, ultimately guiding undifferentiated cells toward malignant transformation. Interestingly, it has been shown that the function of Notch signaling in tumorigenesis could be either oncogenic or anti-proliferative, and the function could be context dependent [11]. Notch signaling has been shown to be anti-proliferative in a limited number of tumor types, including skin cancer, human hepatocellular carcinoma and small cell lung cancer [11–13]. However, most of the studies have shown oncogenic function of Notch in many human carcinomas. In summary, emerging evidence suggest that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer [6,14–17].

Moreover, patients with tumors expressing high levels of Jagged-1 or Notch-1 had a significantly poorer overall survival compared with patients expressing low levels of these genes [18–20]. Jagged-1 was also found to be highly expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues [18]. Fur-

thermore, high Jagged-1 expression in a subset of clinically localized tumors was significantly associated with recurrence, suggesting that Jagged-1 may be a useful marker in distinguishing indolent vs. aggressive prostate carcinomas [18]. Notch signaling pathway has also been reported to cross-talk with multiple oncogenic signaling pathways, such as NF-κB, Akt, Sonic hedgehog (Shh), mTOR, Ras, Wnt, estrogen receptor (ER), androgen receptor (AR), epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) [14,21–25], and thus it is believed that the cross-talk between Notch and other signaling pathways may play critical roles in tumor aggressiveness. The main features of these pathways and cross-talk with Notch signaling have recently been reviewed, and thus the readers who are interested in learning more on the cross-talk between these pathways and Notch pathway are referred published review articles [1,6,7,24,25]. Recently, microRNAs (miRNAs) have been reported to cross-talk with Notch pathway for its regulation [26–30], suggesting that the post-transcriptional and/or translational regulation of genes by miRNAs are becoming critically important. Therefore, in the following sections, we have attempted to summarize the functional role of miRNAs in Notch signaling pathway.

2. miRNAs

In recent years, a large body of literature has emerged documenting the biological significance of miRNAs in tumor progression [31–33]. Over 4500 miRNAs have been identified in vertebrates, flies, worms, plants and viruses after the first miRNA, which was discovered in 1993 while studying Caenorhabditis elegans [34]. It is well known that miRNAs work as integral players in cancer biology. The miRNAs elicit their regulatory effects in post-transcriptional regulation by binding to the 3′ untranslated region (3′ UTR) of target messenger RNA (mRNA). Either perfect or near perfect complimentary base pairing results in the degradation of the mRNA, while partial base pairing leads to translational inhibition to functional proteins [35]. The miRNAs have been implicated in a wide array of cell functions in many normal biological processes, including cell proliferation, differentiation, apoptosis, and stress resistance [36]. It has also been shown that miRNAs are key players in human cancer. The reason why miRNAs are connected with cancer is that miRNAs are involved in the biological processes of cell proliferation and apoptosis, the two intimately linked processes that are critically involved in the development and progression of human malignancies. It has been reported that there are aberrant expression of miRNAs when comparing various types of cancer with normal tissues [37]. It is very important to note that some miRNAs are thought to have oncogenic activity while others have tumor suppressor activity as indicated earlier. Oncogenic miRNAs are up-regulated in cancer and contribute to its pathology through various mechanisms such as targeting tumor suppressor genes. In contrast to the oncogenic miRNAs, other miRNAs are considered to have tumor suppressor activity and are down-regulated in cancer [38,39]. However, these distinctions may not be so strict,

suggesting that some miRNAs may express either activity, depending on the biological context and tissue type.

Recent studies also suggest that miRNAs could have diagnostic, prognostic, and therapeutic value. For example, up-regulation of miR-21 is strongly associated with both a high Ki-67 proliferative index and the presence of liver metastasis [40]. High expression of miR-196a-2 had a median survival of 14.3 months compared with a median of 26.5 months for those with low expression in pancreatic cancer [41], suggesting that miR-196a-2 could be important predictor of survival. Moreover, high expression of miR-15b was significantly associated with poor prognosis and tumorigenesis in melanoma [42]. Furthermore, Patients whose liver tumors had low miR-26 expression had shorter overall survival [43]. Many other published papers showed that miRNAs expression profiling not only can be used in diagnosis, but can also be used as prognostic markers in cancer [37]. Although the research studies for the role of miRNAs in cancer have exploded in recent years, the question remains whether the alteration in miRNAs expression could be ascertained as the cause or the consequence of cancer development [37]. It is not clear for the specific targets and functions of miRNAs although there are several excellent review articles published documenting the role of miRNAs in human cancers [31–37,44], and thus we will not discuss the functions of miRNAs in cancers in this article rather we will present evidence regarding the cross-talk regulation of Notch and miRNAs in cancer development and progression.

3. Cross-talk between Notch and miRNAs

Recently, it has been reported that miRNAs play critical roles in Notch signaling pathway. Several miRNAs have been shown to cross-talk with Notch pathway. However, the role of miRNAs in the Notch pathway remains unclear. Therefore, in this article, we will discuss the effect of miRNAs in the Notch signaling pathway and their cross-talk in tumor development and progression.

3.1. miR-1

It has been well known that some miRNAs have tumor suppressor activity and are down-regulated in cancer. One such miRNA which belongs to tumor suppressor group is the miR-1. In several studies investigating the expression levels of miR-1, the authors have found that the miR-1 was markedly reduced in primary human hepatocellular carcinoma (HCC), prostate cancer, head and neck, and lung cancer [45–49]. Datta et al. have shown that ectopic expression of miR-1 inhibited HCC cell growth and reduced clonogenic survival [47]. In prostate cancer cell lines, transfection with *miR-1* represses the expression of its target genes exportin-6 and protein tyrosine kinase 9 [45]. Nasser et al. reported that re-expression of miR-1 in lung cancer cells reversed their tumorigenic properties, including growth, migration, clonogenic survival, and tumor formation in nude mice. The anti-tumor effect of miR-1 in lung cancer may be mediated through down-regulation of oncogenic targets, such as MET, Pim-1, FoxP1, and HDAC-4. Further, ectopic miR-1 expression was found to induce apoptosis in lung cancer cells in response to the potent anticancer drug doxorubicin, suggesting that miR-1 has potential therapeutic application against lung cancers [48]. Interestingly, the exon 1 and intron 1 of miR-1-1 was methylated in HCC cell lines and in primary human HCC [47]. Recently, it has been reported that miR-1 regulated Notch signaling pathway. Kwon et al. reported that miR-1 directly targets the Notch ligand delta in *Drosophila* for repression [50]. Recently, it has also been found that Dll-1 protein levels are negatively regulated by miR-1 in mouse embryonic stem cells [51]. These results suggest that miR-1 could regulate the Notch signaling pathway; however further in-depth research is needed in order to fully understanding how miR-1 regulate the Notch pathway.

3.2. miR-34

Another important miRNA is miR-34, which has been found to participate in p53 and Notch pathways regulation consistent with tumor suppressor activity [29,52]. In mammalians, the miR-34 family is composed of three processed miRNAs: miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c share a common primary transcript. It has been reported that the expression of miR-34a was lower or undetectable in pancreatic cancer, osteosarcoma, breast cancer and non-small cell lung cancer [53–56]. Recently, the inactivation of miR-34a was identified in cell lines derived from some tumors including lung, breast, colon, kidney, bladder, pancreas and melanoma [57]. More recently, the inactivation of miR-34b/c due to CpG methylation was found in malignant melanoma, colorectal cancer, and oral squamous cell carcinoma [58–60]. Moreover, lower levels of miR-34a expression was correlated with higher probability of relapse in non-small cell lung cancer (NSCLC), suggesting that miR-34a could work as a novel prognostic marker in NSCLC patients [61]. All published data to-date suggests that the inactivation of the miR-34 is a common event in human malignancies.

The reports from several groups have shown that the members of the miR-34 family could direct p53 signaling. Expectedly, ectopic miR-34 inhibited cell proliferation, colony formation, and caused a cell cycle arrest in the G1 phase [53,62]. Moreover, re-expression of miR-34a induced apoptotic cell death [52]. It has been suggested that miR-34-mediated apoptosis could be suppressed by inactivation of p53 gene. It was also documented that miR-34a could target several mRNAs, such as SIRT1, Bcl-2, N-myc, cyclin D1, leading to translational repression of these genes [53,63,64]. Recently, Li et al. reported that transfection of miR-34a to glioma cells down-regulated the protein expression of Notch-1, Notch-2, and CDK6 [26]. More recently, Ji et al. reported that human gastric cancer cells with miR-34 restoration reduced the expression of target gene Notch [28]. Very recently, the same group reported that Notch-1 and Notch-2 is downstream genes of miR-34 in pancreatic cancer cells. They found that restoration of miR-34 expression in the pancreatic cancer cells down-regulated Notch-1 and Notch-2 [29]. They also re-

ported that pancreatic cancer stem cells are enriched with tumor-initiating cells or cancer stem cells with high levels of Notch-1/2 and loss of miR-34. These results suggested that miR-34 may be involved in pancreatic cancer stem cell self-renewal, potentially via the direct modulation of downstream target Notch [29]. Taken together, it may be possible to restore miR-34 function for cancer therapeutic for which novel and innovative research is warranted.

3.3. miR-146

The miR-146 was previously reported to function as novel negative regulators that help to fine-tune the immune response. Konstantin et al. described the role for miR-146 in the control of Toll-like receptor and cytokine signaling through a negative feedback regulation loop involving inhibition of TNF receptor-associated factor 6 protein and IL-1 receptor-associated kinase 1 levels [65]. Recently, it has been found that miR-146a/b acts as terminal transducers of TLR4 signaling by targeting NF-κB activation by TLR4 [66]. They also demonstrated a decrease in miR-146b in adult T-cell Leukemia cells. The decrease in miR-146b may lead to increased inflammation and decreased T-reg functions, resulting in leukemia [66]. Very recently, miR-146a has been found to have the strongest predictive accuracy for stratifying prognostic groups and have also shown superiority in predicting overall survival in lung squamous cell carcinoma [67]. The miR-146 has been reported to cross-talk with breast cancer metastasis suppressor 1 (BRMS1), a predominantly nuclear protein that inhibits metastasis without blocking orthotopic tumor growth. Specifically, BRMS1 significantly up-regulates miR-146a and miR-146b in breast cancer cells. Transduction of miR-146a or miR-146b into breast cancer cells decreased expression of epidermal growth factor receptor, down-regulated NF-κB activity, inhibited migration and invasion in vitro, and suppressed lung metastasis in experimental xenograft models [68,69]. These provided experimental support suggesting that the modulation in the levels of miR-146 could have therapeutic value in inhibiting breast cancer metastasis. Very recently, miR-146a was found to regulate Numb in C2C12 cells [27], which is interesting because Numb is known to regulate Notch signaling negatively through interaction with Notch and the subsequent ubiquitin-mediated protein degradation. Indeed, Notch activation and the loss of Numb expression were found in a large proportion of breast carcinomas [70,71]. It has been reported that over-expression of Notch-1 stimulates NF-κB activity in several cancer cell lines [72] and since miR-146 also regulate NF-κB activity, it clearly suggest that miR-146 could regulate NF-κB through Notch mediated signaling pathway. However, the role of miR-146 in Notch signaling pathway need further innovative investigations.

3.4. miR-199

It has been reported that miR-199a was down-modulated in ovarian cancer [73]. Murakami et al. also found that miR-199a was down-regulated in hepatocellular cancer. Moreover, they found that over-expression of miR-

199a can introduce cell cycle arrest in G2/M phase [74]. Recently, It was reported that miR-199a and miR-199b were down-regulated after 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent tobacco-specific carcinogen, treated rats up to 20 weeks [75]. Very recently, miR-199b-5p was seen to be a regulator of the Notch pathway through its targeting of the transcription factor Hes-1 in medulloblastoma (MB) tumors. Inhibition of Hes-1 by miR-199b-5p negatively regulated the MB cell growth. Moreover, over-expression of miR-199b-5p decreased the MB stem-like cells (CD133+) and also blocked expression of several cancer stem-cell genes. Further, the expression of miR-199b-5p in the non-metastatic cases was significantly higher than in the metastatic cases. The patients with high levels of miR-199b expression showed a better overall survival [30]. These results clearly suggest that miR-199 family could be very important in the regulation of multiple signaling pathways including Notch, and thus further in-depth studies are needed in order to clarify the biological significance and mechanisms on how miR-199 can regulate the Notch signaling pathway in human cancers.

3.5. miR-200

The microRNA-200 family has five members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429. The miR-200c was down-regulated in benign or malignant hepatocellular tumors [76]. It has been shown that three miR-200 miRNAs (miR-200a, miR-200b and miR-429) are significantly associated with cancer recurrence and overall survival in ovarian tumors [77]. Recently many studies have shown that the miR-200 family regulates epithelial–mesenchymal transition (EMT) by targeting zinc-finger E-box binding homeobox 1 (ZEB1) and ZEB2 [78–81]. EMT is a process by which epithelial cells undergo remarkable morphological changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype. We have found that PDGF-D over-expression led to the acquisition of EMT phenotype in PC-3 prostate cells (PC3 PDGF-D cells) consistent with loss of miR-200 expression, and that the re-expression of miR-200b in PC3 PDGF-D cells led to the reversal of the EMT phenotype, which was associated with the down-regulation of ZEB1, ZEB2, and Snail2 expression [82]. Moreover, transfection of PC3 PDGF-D cells with miR-200b inhibited cell migration and invasion with concomitant repression of cell adhesion to the culture surface and cell detachment [82]. We also found that miR-200a, miR-200b, miR-200c, and many members of the tumor suppressor let-7 family were down-regulated in gemcitabine-resistant (GR) pancreatic cancer cells, which show the acquisition of EMT phenotype [83]. Furthermore, we have shown that miR-200 family regulates the expression of ZEB1, slug, E-cadherin, and vimentin, and thus the re-expression of miR-200 could be useful for the reversal of EMT phenotype to mesenchymal-to-epithelial transition [83]. We have found that the expression of both mRNA and protein levels of Notch-1 to -4, Dll-1, Dll-3, Dll-4, Jagged-2 as well as Notch downstream targets, such as Hes and Hey, were significantly higher in PC3 PDGF-D cells (unpublished data). More

importantly, we found that Notch-1 could be one of miR-200b targets because over-expression of miR-200b significantly inhibited Notch-1 expression (unpublished data). However, how the miR-200b regulates Notch gene expression will certainly require further in-depth investigations.

4. miRNA as targets by natural agents

Emerging experimental studies have shown that targeting miRNA could be a novel strategy for cancer prevention and/or treatment. There are several strategies that could be used for targeting the regulation of miRNAs, which could be useful tool for the inhibition of tumor progression and, as such, could be useful for therapy. One potential strategy could be the inactivation of oncogenic miRNAs. It has been found that 2′-O-methyl oligonucleotides or locked nucleic acid-modified oligonucleotides can block miRNA function. For example, using this anti-sense oligonucleotide, one could significantly decrease the activity of miR-21 as compared to control oligonucleotides [84]. Another strategy is to restore down-regulated miRNAs that function as tumor suppressors, such as let-7. It has been shown that over-expression of let-7 by using exogenously transfected pre-let-7 RNAs consistently showed reduction in the number of proliferating cells in lung and liver cancer cell lines [85]. This finding clearly suggests the possibility of restoration of tumor suppressor miRNAs toward cancer therapy. A third possible strategy could be the use of "natural agents" to target miRNAs that are known to contribute in the processes of tumor development and progression.

To that end, recent studies have shown that "natural agents" including curcumin, isoflavone, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), EGCG, and others could alter the expression of specific miRNAs, which may lead to increased sensitivity of cancer cells to conventional therapeutic agents, and thereby may result in the inhibition of tumor growth. We have found that alteration in the expression of miRNAs could be achieved by treating cancer cells with DIM or isoflavone. We have shown that treatment of Panc-1 or Colo-357 cells with B-DIM or genistein (isoflavone) showed decreased expression of the oncogenic miRNA such as miR-17, miR-20a, miR-106a, and increased the expression of the tumor suppressor miRNAs such as let-7, miR-16-1 [86]. Our results clearly suggest that "natural agents" may exhibit their anti-tumor effects through the regulation of miRNAs. Further support to this statement comes from findings reported by Sun et al. showing that curcumin could alter specific miRNA expression in human pancreatic cancer cells especially showing up-regulation of miR-22. They also found that up-regulation of miR-22 expression by curcumin in pancreatic cancer cells suppressed the expression of its target genes SP1 transcription factor (SP1) and estrogen receptor 1 (ESR1) [87]. Melkamu et al. reported that I3C can inhibit the expression of several oncogenic miRNAs, such as miR-21, miR-31, miR-130a, miR-146b, and miR-377 in vinyl carbamate treated animals. Further investigation showed that I3C up-regulated PTEN tumor suppressor gene though inhibition of miR-21 [49]. Tsang et al. recently reported that EGCG treatment could up-regulate the expressions of miR-16 in human
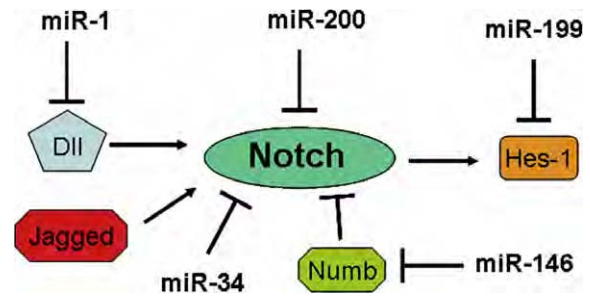

Fig. 2. Diagram of roles of miRNA in the Notch pathway.

hepatocellular carcinoma cells [88]. We also found that the expression of miR-200 and let-7 families could be up-regulated in gemcitabine resistant cells by DIM or isoflavone treatment as indicated above. Our results also showed that DIM treatment cause down-regulation of ZEB1, slug, and vimentin, and the morphologic reversal of EMT to epithelial morphology [83]. Considering the non-toxic characteristics of "natural agents", one could speculate that targeting miRNAs by "natural agents" could be a novel and safer approach for the prevention of tumor progression and/or treatment of human malignancies in the future.

5. Concluding remarks

In conclusion, we believe that the deregulation of miRNAs plays important roles in the development and progression of human cancers, and during the acquisition of EMT phenotype that are in part associated with the formation and maintenance of cancer stem cells (CSCs). Importantly, miRNAs have been characterized as biomarkers for diagnosis and prognosis, as well as targets for cancer therapy. Although emerging evidence suggest an interrelationship between miRNAs and Notch signaling pathway (Fig. 2), further research is warranted to ascertain the value of specific miRNA in the regulation of Notch signaling in order to exploit preventive and therapeutic strategies. Due to the non-toxic nature of "natural agents", we believe that targeting miRNAs by "natural agents" combined with conventional chemotherapeutics could be a novel and safer approach for the treatment of cancer. The findings reported in the short review article are very interesting; however, further investigations are needed in order to elucidate the roles of these and numerous other miRNAs that could be mechanistically linked with Notch and other cell signaling, and devising novel approaches on how "natural agents" could be useful in combination therapy for the prevention and/or treatment of human malignancies in the future.

Conflict of interest

None declared.

Acknowledgements

References

[1] L. Miele, H. Miao, B.J. Nickoloff, Notch signaling as a novel cancer therapeutic target, Curr. Cancer Drug Targets 6 (2006) 313–323.

[2] R. Kopan, M.X. Ilagan, The canonical Notch signaling pathway: unfolding the activation mechanism, Cell 137 (2009) 216–233.

[3] L. Miele, Notch signaling, Clin. Cancer Res. 12 (2006) 1074–1079.

[4] J. Dufraine, Y. Funahashi, J. Kitajewski, Notch signaling regulates tumor angiogenesis by diverse mechanisms, Oncogene 27 (2008) 5132–5137.

[5] J.L. Li, A.L. Harris, Notch signaling from tumor cells: a new mechanism of angiogenesis, Cancer Cell 8 (2005) 1–3.

[6] P. Rizzo, C. Osipo, K. Foreman, T. Golde, B. Osborne, L. Miele, Rational targeting of Notch signaling in cancer, Oncogene 27 (2008) 5124–5131.

[7] Z. Wang, Y. Li, S. Banerjee, F.H. Sarkar, Exploitation of the Notch signaling pathway as a novel target for cancer therapy, Anticancer Res. 28 (2008) 3621–3630.

[8] Z. Wang, Y. Li, S. Banerjee, F.H. Sarkar, Emerging role of Notch in stem cells and cancer, Cancer Lett. 279 (2009) 8–12.

[9] S. Malinge, S. Izraeli, J.D. Crispino, Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in Down syndrome, Blood 113 (2009) 2619–2628.

[10] R.M. Demarest, F. Ratti, A.J. Capobianco, It's T-ALL about Notch, Oncogene 27 (2008) 5082–5091.

[11] G.P. Dotto, Notch tumor suppressor function, Oncogene 27 (2008) 5115–5123.

[12] V. Sriuranpong, M.W. Borges, R.K. Ravi, D.R. Arnold, B.D. Nelkin, S.B. Baylin, D.W. Ball, Notch signaling induces cell cycle arrest in small cell lung cancer cells, Cancer Res. 61 (2001) 3200–3205.

[13] M. Nicolas, A. Wolfer, K. Raj, J.A. Kummer, P. Mill, N.M. van, C.C. Hui, H. Clevers, G.P. Dotto, F. Radtke, Notch1 functions as a tumor suppressor in mouse skin, Nat. Genet. 33 (2003) 416–421.

[14] Z. Wang, S. Banerjee, Y. Li, K.M. Rahman, Y. Zhang, F.H. Sarkar, Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{kappa}B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells, Cancer Res. 66 (2006) 2778–2784.

[15] Z. Wang, Y. Zhang, Y. Li, S. Banerjee, J. Liao, F.H. Sarkar, Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells, Mol. Cancer Ther. 5 (2006) 483–493.

[16] P.J. Real, A.A. Ferrando, NOTCH inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia, Leukemia 23 (2009) 1374–1377.

[17] L. Strizzi, K.M. Hardy, E.A. Seftor, F.F. Costa, D.A. Kirschmann, R.E. Seftor, L.M. Postovit, M.J. Hendrix, Development and cancer: at the crossroads of Nodal and Notch signaling, Cancer Res. 69 (2009) 7131–7134.

[18] S. Santagata, F. Demichelis, A. Riva, S. Varambally, M.D. Hofer, J.L. Kutok, R. Kim, J. Tang, J.E. Montie, A.M. Chinnaiyan, M.A. Rubin, J.C. Aster, JAGGED1 expression is associated with prostate cancer metastasis and recurrence, Cancer Res. 64 (2004) 6854–6857.

[19] M. Reedijk, S. Odorcic, L. Chang, H. Zhang, N. Miller, D.R. McCready, G. Lockwood, S.E. Egan, High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival, Cancer Res. 65 (2005) 8530–8537.

[20] M. Reedijk, D. Pinnaduwage, B.C. Dickson, A.M. Mulligan, H. Zhang, S.B. Bull, F.P. O'Malley, S.E. Egan, I.L. Andrulis, JAG1 expression is associated with a basal phenotype and recurrence in lymph node-negative breast cancer, Breast Cancer Res. Treat. (2007).

[21] Z. Wang, R. Sengupta, S. Banerjee, Y. Li, Y. Zhang, K.M. Rahman, A. Aboukameel, R. Mohammad, A.P. Majumdar, J.L. Abbruzzese, F.H. Sarkar, Epidermal growth factor receptor-related protein inhibits cell growth and invasion in pancreatic cancer, Cancer Res. 66 (2006) 7653–7660.

[22] Z. Wang, D. Kong, S. Banerjee, Y. Li, N.V. Adsay, J. Abbruzzese, F.H. Sarkar, Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling, Cancer Res. 67 (2007) 11377–11385.

[23] S.M. Chan, A.P. Weng, R. Tibshirani, J.C. Aster, P.J. Utz, Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia, Blood 110 (2007) 278–286.

[24] X. Guo, X.F. Wang, Signaling cross-talk between TGF-beta/BMP and other pathways, Cell Res. 19 (2009) 71–88.

[25] L. Poellinger, U. Lendahl, Modulating Notch signaling by pathway-intrinsic and pathway-extrinsic mechanisms, Curr. Opin. Genet. Dev. 18 (2008) 449–454.

[26] Y. Li, F. Guessous, Y. Zhang, C. Dipierro, B. Kefas, E. Johnson, L. Marcinkiewicz, J. Jiang, Y. Yang, T.D. Schmittgen, B. Lopes, D. Schiff, B. Purow, R. Abounader, MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes, Cancer Res. 69 (2009) 7569–7576.

[27] W. Kuang, J. Tan, Y. Duan, J. Duan, W. Wang, F. Jin, Z. Jin, X. Yuan, Y. Liu, Cyclic stretch induced miR-146a upregulation delays C2C12 myogenic differentiation through inhibition of Numb, Biochem. Biophys. Res. Commun. 378 (2009) 259–263.

[28] Q. Ji, X. Hao, Y. Meng, M. Zhang, J. Desano, D. Fan, L. Xu, Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres, BMC Cancer 8 (2008) 266.

[29] Q. Ji, X. Hao, M. Zhang, W. Tang, M. Yang, L. Li, D. Xiang, J.T. Desano, G.T. Bommer, D. Fan, E.R. Fearon, T.S. Lawrence, L. Xu, MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells, PLoS One 4 (2009) e6816.

[30] L. Garzia, I. Andolfo, E. Cusanelli, N. Marino, G. Petrosino, M.D. De, V. Esposito, A. Galeone, L. Navas, S. Esposito, S. Gargiulo, S. Fattet, V. Donofrio, G. Cinalli, A. Brunetti, L.D. Vecchio, P.A. Northcott, O. Delattre, M.D. Taylor, A. Iolascon, M. Zollo, MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma, PLoS One 4 (2009) e4998.

[31] B.D. Brown, L. Naldini, Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications, Nat. Rev. Genet. 10 (2009) 578–585.

[32] R. Garzon, G.A. Calin, C.M. Croce, MicroRNAs in Cancer, Annu. Rev. Med. 60 (2009) 167–179.

[33] A. Petri, M. Lindow, S. Kauppinen, MicroRNA silencing in primates: towards development of novel therapeutics, Cancer Res. 69 (2009) 393–395.

[34] Y.S. Lee, A. Dutta, MicroRNAs in cancer, Annu. Rev. Pathol. 4 (2009) 199–227.

[35] K.M. Nelson, G.J. Weiss, MicroRNAs and cancer: past, present, and potential future, Mol. Cancer Ther. 7 (2008) 3655–3660.

[36] S.P. O'Hara, J.L. Mott, P.L. Splinter, G.J. Gores, N.F. LaRusso, MicroRNAs: key modulators of posttranscriptional gene expression, Gastroenterology 136 (2009) 17–25.

[37] C.M. Croce, Causes and consequences of microRNA dysregulation in cancer, Nat. Rev. Genet. 10 (2009) 704–714.

[38] S. Deng, G.A. Calin, C.M. Croce, G. Coukos, L. Zhang, Mechanisms of microRNA deregulation in human cancer, Cell Cycle 7 (2008) 2643–2646.

[39] P.P. Medina, F.J. Slack, MicroRNAs and cancer: an overview, Cell Cycle 7 (2008) 2485–2492.

[40] C. Roldo, E. Missiaglia, J.P. Hagan, M. Falconi, P. Capelli, S. Bersani, G.A. Calin, S. Volinia, C.G. Liu, A. Scarpa, C.M. Croce, MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior, J. Clin. Oncol. 24 (2006) 4677–4684.

[41] M. Bloomston, W.L. Frankel, F. Petrocca, S. Volinia, H. Alder, J.P. Hagan, C.G. Liu, D. Bhatt, C. Taccioli, C.M. Croce, MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis, JAMA 297 (2007) 1901–1908.

[42] I. Satzger, A. Mattern, U. Kuettler, D. Weinspach, B. Voelker, A. Kapp, R. Gutzmer, MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma, Int. J. Cancer (2009).

[43] J. Ji, J. Shi, A. Budhu, Z. Yu, M. Forgues, S. Roessler, S. Ambs, Y. Chen, P.S. Meltzer, C.M. Croce, L.X. Qin, K. Man, C.M. Lo, J. Lee, I.O. Ng, J. Fan, Z.Y. Tang, H.C. Sun, X.W. Wang, MicroRNA expression, survival, and response to interferon in liver cancer, N. Engl. J. Med. 361 (2009) 1437–1447.

[44] J. Winter, S. Jung, S. Keller, R.I. Gregory, S. Diederichs, Many roads to maturity: microRNA biogenesis pathways and their regulation, Nat. Cell Biol. 11 (2009) 228–234.

[45] S. Ambs, R.L. Prueitt, M. Yi, R.S. Hudson, T.M. Howe, F. Petrocca, T.A. Wallace, C.G. Liu, S. Volinia, G.A. Calin, H.G. Yfantis, R.M. Stephens,

C.M. Croce, Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer, Cancer Res. 68 (2008) 6162–6170.

[46] G. Childs, M. Fazzari, G. Kung, N. Kawachi, M. Brandwein-Gensler, M. McLemore, Q. Chen, R.D. Burk, R.V. Smith, M.B. Prystowsky, T.J. Belbin, N.F. Schlecht, Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma, Am. J. Pathol. 174 (2009) 736–745.

[47] J. Datta, H. Kutay, M.W. Nasser, G.J. Nuovo, B. Wang, S. Majumder, C.G. Liu, S. Volinia, C.M. Croce, T.D. Schmittgen, K. Ghoshal, S.T. Jacob, Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis, Cancer Res. 68 (2008) 5049–5058.

[48] M.W. Nasser, J. Datta, G. Nuovo, H. Kutay, T. Motiwala, S. Majumder, B. Wang, S. Suster, S.T. Jacob, K. Ghoshal, Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1, J. Biol. Chem. 283 (2008) 33394–33405.

[49] T. Melkamu, X. Zhang, J. Tan, Y. Zeng, F. Kassie, Alteration of microRNA expression in vinyl-carbamate-induced mouse lung tumors and modulation by the chemopreventive agent indole-3-carbinol, Carcinogenesis (2009).

[50] C. Kwon, Z. Han, E.N. Olson, D. Srivastava, MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling, Proc. Natl. Acad. Sci. USA 102 (2005) 18986–18991.

[51] K.N. Ivey, A. Muth, J. Arnold, F.W. King, R.F. Yeh, J.E. Fish, E.C. Hsiao, R.J. Schwartz, B.R. Conklin, H.S. Bernstein, D. Srivastava, MicroRNA regulation of cell lineages in mouse and human embryonic stem cells, Cell Stem Cell 2 (2008) 219–229.

[52] H. Hermeking, The miR-34 family in cancer and apoptosis, Cell Death Differ. (2009).

[53] G.T. Bommer, I. Gerin, Y. Feng, A.J. Kaczorowski, R. Kuick, R.E. Love, Y. Zhai, T.J. Giordano, Z.S. Qin, B.B. Moore, O.A. MacDougald, K.R. Cho, E.R. Fearon, P53-mediated activation of miRNA34 candidate tumor-suppressor genes, Curr. Biol. 17 (2007) 1298–1307.

[54] T.C. Chang, E.A. Wentzel, O.A. Kent, K. Ramachandran, M. Mullendore, K.H. Lee, G. Feldmann, M. Yamakuchi, M. Ferlito, C.J. Lowenstein, D.E. Arking, M.A. Beer, A. Maitra, J.T. Mendell, Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis, Mol. Cell 26 (2007) 745–752.

[55] C. He, J. Xiong, X. Xu, W. Lu, L. Liu, D. Xiao, D. Wang, Functional elucidation of MiR-34 in osteosarcoma cells and primary tumor samples, Biochem. Biophys. Res. Commun. 388 (2009) 35–40.

[56] M. Kato, T. Paranjape, R.U. Muller, S. Nallur, E. Gillespie, K. Keane, A. Esquela-Kerscher, J.B. Weidhaas, F.J. Slack, The mir-34 microRNA is required for the DNA damage response in vivo in C. elegans and in vitro in human breast cancer cells, Oncogene 28 (2009) 2419–2424.

[57] D. Lodygin, V. Tarasov, A. Epanchintsev, C. Berking, T. Knyazeva, H. Korner, P. Knyazev, J. Diebold, H. Hermeking, Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer, Cell Cycle 7 (2008) 2591–2600.

[58] K. Kozaki, I. Imoto, S. Mogi, K. Omura, J. Inazawa, Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer, Cancer Res. 68 (2008) 2094–2105.

[59] A. Lujambio, G.A. Calin, A. Villanueva, S. Ropero, M. Sanchez-Cespedes, D. Blanco, L.M. Montuenga, S. Rossi, M.S. Nicoloso, W.J. Faller, W.M. Gallagher, S.A. Eccles, C.M. Croce, M. Esteller, A microRNA DNA methylation signature for human cancer metastasis, Proc. Natl. Acad. Sci. USA 105 (2008) 13556–13561.

[60] M. Toyota, H. Suzuki, Y. Sasaki, R. Maruyama, K. Imai, Y. Shinomura, T. Tokino, Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer, Cancer Res. 68 (2008) 4123–4132.

[61] E. Gallardo, A. Navarro, N. Vinolas, R.M. Marrades, T. Diaz, B. Gel, A. Quera, E. Bandres, J. Garcia-Foncillas, J. Ramirez, M. Monzo, miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer, Carcinogenesis (2009).

[62] V. Tarasov, P. Jung, B. Verdoodt, D. Lodygin, A. Epanchintsev, A. Menssen, G. Meister, H. Hermeking, Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest, Cell Cycle 6 (2007) 1586–1593.

[63] M. Yamakuchi, M. Ferlito, C.J. Lowenstein, MiR-34a repression of SIRT1 regulates apoptosis, Proc. Natl. Acad. Sci. USA 105 (2008) 13421–13426.

[64] N.R. Christoffersen, R. Shalgi, L.B. Frankel, E. Leucci, M. Lees, M. Klausen, Y. Pilpel, F.C. Nielsen, M. Oren, A.H. Lund, p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC, Cell Death Differ. (2009).

[65] K.D. Taganov, M.P. Boldin, K.J. Chang, D. Baltimore, NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses, Proc. Natl. Acad. Sci. USA 103 (2006) 12481–12486.

[66] M. Bellon, Y. Lepelletier, O. Hermine, C. Nicot, Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia, Blood 113 (2009) 4914–4917.

[67] M. Raponi, L. Dossey, T. Jatkoe, X. Wu, G. Chen, H. Fan, D.G. Beer, MicroRNA classifiers for predicting prognosis of squamous cell lung cancer, Cancer Res. 69 (2009) 5776–5783.

[68] D.R. Hurst, M.D. Edmonds, G.K. Scott, C.C. Benz, K.S. Vaidya, D.R. Welch, Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis, Cancer Res. 69 (2009) 1279–1283.

[69] D. Bhaumik, G.K. Scott, S. Schokrpur, C.K. Patil, J. Campisi, C.C. Benz, Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells, Oncogene (2008).

[70] S. Stylianou, R.B. Clarke, K. Brennan, Aberrant activation of notch signaling in human breast cancer, Cancer Res. 66 (2006) 1517–1525.

[71] S. Pece, M. Serresi, E. Santolini, M. Capra, E. Hulleman, V. Galimberti, S. Zurrida, P. Maisonneuve, G. Viale, P.P. Di Fiore, Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis, J. Cell Biol. 167 (2004) 215–221.

[72] C. Osipo, T.E. Golde, B.A. Osborne, L.A. Miele, Off the beaten pathway: the complex cross talk between Notch and NF-kappaB, Lab. Invest. 88 (2008) 11–17.

[73] M.V. Iorio, R. Visone, L.G. Di, V. Donati, F. Petrocca, P. Casalini, C. Taccioli, S. Volinia, C.G. Liu, H. Alder, G.A. Calin, S. Menard, C.M. Croce, MicroRNA signatures in human ovarian cancer, Cancer Res. 67 (2007) 8699–8707.

[74] Y. Murakami, T. Yasuda, K. Saigo, T. Urashima, H. Toyoda, T. Okanoue, K. Shimotohno, Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues, Oncogene 25 (2006) 2537–2545.

[75] S. Kalscheuer, X. Zhang, Y. Zeng, P. Upadhyaya, Differential expression of microRNAs in early-stage neoplastic transformation in the lungs of F344 rats chronically treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, Carcinogenesis 29 (2008) 2394–2399.

[76] Y. Ladeiro, G. Couchy, C. Balabaud, P. Bioulac-Sage, L. Pelletier, S. Rebouissou, J. Zucman-Rossi, MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations, Hepatology 47 (2008) 1955–1963.

[77] X. Hu, D.M. Macdonald, P.C. Huettner, Z. Feng, N. El, J.K. Schwarz, D.G. Mutch, P.W. Grigsby, S.N. Powell, X. Wang, A miR-200 microRNA cluster as prognostic marker in advanced ovarian cancer, Gynecol. Oncol. 114 (2009) 457–464.

[78] U. Burk, J. Schubert, U. Wellner, O. Schmalhofer, E. Vincan, S. Spaderna, T. Brabletz, A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells, EMBO Rep. 9 (2008) 582–589.

[79] P.A. Gregory, A.G. Bert, E.L. Paterson, S.C. Barry, A. Tsykin, G. Farshid, M.A. Vadas, Y. Khew-Goodall, G.J. Goodall, The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1, Nat. Cell Biol. 10 (2008) 593–601.

[80] M. Korpal, E.S. Lee, G. Hu, Y. Kang, The miR-200 family inhibits epithelial–mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2, J. Biol. Chem. 283 (2008) 14910–14914.

[81] S.M. Park, A.B. Gaur, E. Lengyel, M.E. Peter, The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2, Genes Dev. 22 (2008) 894–907.

[82] D. Kong, Y. Li, Z. Wang, S. Banerjee, A. Ahmad, H.R. Kim, F.H. Sarkar, MiR-200 regulates PDGF-D-mediated epithelial–mesenchymal transition, adhesion, and invasion of prostate cancer cells, Stem Cells 27 (2009) 1712–1721.

[83] Y. Li, T.G. VandenBoom, D. Kong, Z. Wang, S. Ali, P.A. Philip, F.H. Sarkar, Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells, Cancer Res. 69 (2009) 6704–6712.

[84] G. Meister, M. Landthaler, Y. Dorsett, T. Tuschl, Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing, RNA 10 (2004) 544–550.

[85] C.D. Johnson, A. Esquela-Kerscher, G. Stefani, M. Byrom, K. Kelnar, D. Ovcharenko, M. Wilson, X. Wang, J. Shelton, J. Shingara, L. Chin, D.

Brown, F.J. Slack, The let-7 microRNA represses cell proliferation pathways in human cells, Cancer Res. 67 (2007) 7713–7722.

[86] T.G. Vandenboom Ii, Y. Li, P.A. Philip, F.H. Sarkar, MicroRNA and cancer: tiny molecules with major implications, Curr. Genom. 9 (2008) 97–109.

[87] M. Sun, Z. Estrov, Y. Ji, K.R. Coombes, D.H. Harris, R. Kurzrock, Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells, Mol. Cancer Ther. 7 (2008) 464–473.

[88] W.P. Tsang, T.T. Kwok, Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells, J. Nutr. Biochem. (2009).

Mini-review

Emerging role of Notch in stem cells and cancer

Zhiwei Wang, Yiwei Li, Sanjeev Banerjee, Fazlul H. Sarkar *

Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, 9374 Scott Hall, 540 E Canfield, Detroit, MI 48201, United States

ARTICLE INFO

ABSTRACT

The Notch signaling pathway is known to be responsible for maintaining a balance between cell proliferation and death and, as such, plays important roles in the formation of many types of human tumors. Recently, Notch signaling pathway has been shown to control stem cell self-renewal and multi-potency. As many cancers are thought to be developed from a number of cancer stem-like cells, which are also known to be linked with the acquisition of epithelial-mesenchymal transition (EMT); and thus suggesting an expanding role of Notch signaling in human tumor progression.

© 2008 Elsevier Ireland Ltd. All rights reserved.

1. Notch pathway

Notch signaling is involved in cell proliferation and apoptosis which affects the development and function of many organs. Notch genes encode proteins which can be activated by interacting with a family of its ligands. Upon activation, Notch is cleaved, releasing intracellular domain of the Notch (ICN) through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase. The first cleavage is mediated by TACE, which cleaves the receptor in the extracellular domain. The released extracellular domain is then trans-endocytosed by the ligand-expressing cell. The second cleavage caused by the γ-secretase activity of a multi-protein complex consisting of presenilin, nicastrin, etc. releasing the intracellular fragment of Notch (ICN) which is then ready to be translocated into the nucleus for transcriptional activation of Notch target genes [1,2]. Therefore, inhibiting γ-secretase activity could prevent the cleavage of the Notch receptor, thus blocking Notch signal transduction.

In the absence of ICN cleavage, transcription of Notch target genes is inhibited by a repressor complex mediated by the CSL (CBF1, Suppressor of Hairlesss or Lag-1). When ICN enters the nucleus, it recruits transcription activators to the CSL complex and converts it from a transcriptional repressor into an activator, which activates the Notch target genes [1,2]. To date, four vertebrate Notch genes have been identified: Notch-1-4. In addition, five ligands, such as Dll-1, Dll-3, Dll-4, Jagged-1 and Jagged-2, have been found in mammals. A few Notch target genes have also been identified, some of which are dependent on Notch signaling in multiple tissues, while others are tissue specific. Notch target genes include the Hes-1 (Hairy enhance of split-1), Nuclear factor-κB (NF-κB), Cyclin D1, c-myc, etc. [1]. Multiple oncogenic pathways, such as NF-κB, Akt, Sonic hedgehog (Shh), mammalian target of rapamycin (mTOR), Ras, Wnt, epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) signaling have been reported to cross-talk with Notch pathway and, thus it is generally believed that the cross-talk between Notch and other signaling pathways plays important roles in cancer stem cells and tumor aggressiveness [3–9] as discussed below.

2. Stem cells and cancer stem-like cells

Recent results have indicated that Notch signaling pathway contributes to cancer progression by activating

---

* Corresponding author. Tel.: +1 313 576 8327; fax: +1 313 576 8389.
E-mail address: fsarkar@med.wayne.edu (F.H. Sarkar).

transcription factors that promote cell survival, motility, and tumor angiogenesis. Recently, many reports describing molecular connections between Notch regulated transcription factors and pathways known to control stem cell function, further suggesting a new mechanism claiming that Notch may drive tumor growth through the generation or expansion of tumor-initiating cells or cancer stem-like cells [10–15].

Stem cells are characterized by their capacity to self-renewal, and differentiate into the full spectrum of cells forming a particular organism or tissue. Stem cells consists three major types: embryonic, germinal, and somatic [16]. The inner cell mass of the blastocyst generates embryonic stem cells. The embryonic stem cells have the capacity to generate any cell types in the mature organism, and also have unlimited capacity to replicate. Germinal stem cells come from the germinal layer of the embryo and they differentiate to generate specific organs. Somatic stem cells have the capacity to self-renew and differentiate into many types of cells that are the characteristics of a specific organ or tissue [16].

Stem cells often stay at locations which are called stem cell niches. Specifically, stem cell niches are defined as particular locations or microenvironments that maintain the combined properties of stem cell self-renewal and multipotency. There are three kinds of stem cell niches such as simple niches, complex niches, and storage niches [17]. Simple niches are defined as a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing. In general, a stem cell is associated with a permanent partner cell through an adherent junction. The stem cells divide to release another stem cell and a differentiating daughter cell. Complex niches mean that two or more different stem cells are supported by one or more different partner cells. Their activity is coordinately regulated to generate multiple progeny of cells by niche regulatory signals. Storage niches are locations that quiescent stem cells are maintained in a niche until activated by external signals to divide and migrate [17].

A combination of genetic and molecular analyses has identified many factors that support stem cell niche that also control stem cell identity. These factors include components of Notch, Wnt, and Sonic hedgehog (Shh) signaling pathway [18–20]. It has been suggested that the capability of a tumor to grow and propagate is due to a small subset of cells within the tumor, termed cancer stem-like cells (CSCs). Although the concept of "cancer stem-like cell" was first proposed more then 150 years ago, it has become more attractive recently due to advances in stem cell biology, leading to the identification of these cells from a wide variety of human cancers [21]. CSCs have been identified and isolated from tumors of the hematopoietic system, breast, lung, prostate, colon, brain, head and neck, and pancreas [22]. CSCs are able to self-renew, differentiate, and regenerate to a phenotypic cells of the original tumor when implanted into the severe combined immunodeficient mouse [22]. The concept of CSCs have generated considerable attention in recent years, which is likely to provide clear understanding of tumor biology, and for designing novel therapy targeted toward these cells for the complete eradication of tumor growth.

3. Notch pathway in stem cells

It has been reported that altered Notch signaling affects the function of a variety of mammalian stem cells such as hematopoietic, intestinal, and skin stem cells, and intestinal stem cells in Drosophila and germ stem cells in C. elegans [17,19,23]. Recently, Ohlstein et al. reported that intestinal stem cells (ISCs) in adult Drosophila midgut containing high levels of cytoplasmic Delta-rich vesicles activate the canonical Notch pathway and down-regulate Delta within their daughters, a process that programs these daughters to become enterocytes. However, ISCs expressing little vesiculate Delta, or being impaired in Notch signaling, specify their daughters to become enteroendocrine cells. Thus, ISCs control daughter cell fate by modulating Notch signaling over time, suggesting that ISCs actively coordinate cell production with local tissue requirements regulated by Notch signaling pathway [24].

The Drosophila germline stem cells (GSCs) reside in a somatic cell niche. Ward et al. showed that Notch activation can induce the expression of niche-cell markers even in an adult fly. Over-expression of Delta in the germline, or activated Notch in the somatic cells, results in extra niche cells, up to 10-fold over the normal number. In turn, these ectopic niche cells induce ectopic GSCs [25]. In addition, Notch signaling is required for hypoxia promoting the undifferentiated cell state in various stem and precursor cell populations. Hypoxia blocks neuronal and myogenic differentiation in a Notch-dependent manner. Hypoxia activates Notch-responsive promoters and increases expression of Notch directed downstream genes such as Hes-1 and Hey-2. The ICN interacts with HIF-1α, and HIF-1α is recruited to Notch-responsive promoters upon Notch activation under hypoxic conditions. These data provide molecular insights into how Notch controls the cellular differentiation status [26] and the maintenance of stem cells.

Notch signaling is also critical for the maintenance of undifferentiated stem and progenitor cell populations in the mammalian intestinal crypt and also influences differentiation of mature enterocytes [10]. Wilson and Radtke suggested that many of the general Notch functions such as stem cell gate keeper, influencing binary cell fate decisions or induction of terminal differentiation processes exists in invertebrates and self-renewing organ systems of mammals [10]. In the intestine, Notch plays a gate-keeper function for crypt progenitor cells. Notch also seems to control binary cell fate decisions of cells that have to choose between the secretory and adsorptive lineages, most likely by Notch induced expression of Hes-1 [10,27].

4. Notch pathway in cancer stem cells

Cancer stem cells constitute a small subset of cancer cells being a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These cells are characterized by specific stem cell markers: antigens, molecules and signaling pathways. The pathways that regulate self-renewal and cell fate in these systems are beginning to be elucidated. Transcription factors and molecules associated with oncogenesis, such as Notch, NF-κB,

B lymphoma Mo-MLV insertion region (Bmi-1), Wnt, Sonic hedgehog pathways, are active only in a small minority of cancer cells although they may play key roles in determining the biological behavior of a tumor [28]. Katoh reported that the balance between Wnt-FGF-Notch and BMP-Hedgehog signaling networks is important for the maintenance of homeostasis among stem and progenitor cells. Disruption of the stem cell signaling network results in congenital diseases and cancer [29]. In addition to pathways such as Wnt, Notch and Hedgehog are known to regulate self-renewal of normal stem cells, while tumor suppressor genes such as PTEN (phosphatase and tensin homolog on chromosome 10) and P53 (tumor protein p53) are also reported to have implications in the regulation of cancer stem cell self-renewal [21].

It has been reported that Notch signaling plays a critical role in normal human mammary development by acting on both stem cells and progenitor cells, suggesting that abnormal Notch signaling may contribute to mammary carcinogenesis by deregulating the self-renewal of normal mammary stem cells [30]. Dontu et al. observed a 10-fold increase in secondary mammosphere formation upon addition of a Notch-activating DSL peptide, suggesting that Notch signaling can act on mammary stem cells to promote self-renewal. Notch signaling was also found to act on multi-potent progenitor cells, facilitating myoepithelial lineage-specific commitment and proliferation. Phillips et al. reported that cancer stem cells can be identified by phenotypic markers and their fate is controlled by the Notch pathway in breast cancer. Recombinant human erythropoietin receptor increased the numbers of stem cells and self-renewing capacity in a Notch-dependent fashion by induction of Jagged-1. Inhibitors of the Notch pathway blocked this effect, suggesting the mechanistic role of Notch signaling in the maintenance of cancer stem-like cell phenotype [31]. Farnie and Clarke also provided evidence for breast cancer stem cells, and their studies have consistently shown that stem-like cells and breast cancer initiating populations can be enriched using cell surface makers CD44+/CD24− and, as such, these cells showed up-regulated genes including Notch that are known to contribute to cancer stem-like cells characteristics [32].

A small population of cancer stem cells obtained from brain tumors could form neurospheres, which have the capacity for self-renewal, and are able to differentiate into diverse populations including neuronal, astrocytic and oligodendroglial cells when dissociated in single cell suspension [33,34]. The small population of stem cells, also termed "side population" (SP), has been found in long-term culture of glioma cell lines such as human U87-MG and U373-MG [35]. SP stem cells have elevated chemo-resistance because of the high expression levels of drug-transporter proteins such as ABCG2, an ATP-binding cassette half-transporter associated with multi-drug resistance. Furthermore, ABCG2 expression is also associated with proliferation, and the ABCG2 positive cells preferentially expresses several "stemness" genes such as Notch-1 [35].

Since gliomas are the most common tumors of the central nervous system and an important cause of mental impairment and death, emerging research has been intensified for this disease. Increasing body of evidence suggests that gliomas may rise from cancer stem cells sharing with neural stem cells the capacity of self-renewal and multipotency. Interestingly, Notch signaling has been shown to be involved in promoting the formation of cancer stem cell-like cells in human glioma. Notch signaling is appears to be essential for the maintenance of neural stem cells (NSC), by enhancing the NSC self-renewal and by inhibiting its differentiation into neuronal and glial progenitor [36–38]. In addition, Notch signaling prevents nestin degradation during neural stem cell differentiation, by a mechanism that involves ubiquitin-proteosome pathways [39]. Recent data also suggest that Notch signaling can directly up-regulate nestin expression in gliomas, and cooperate with K-ras to lead to their expansion within the subventricular zone and retention of proliferation and nestin expression [40]. Recently, Zhang et al. reported that over-expression of Notch-1 in SHG-44 glioma cells promoted the growth and the colony-forming activity of these cells. Moreover, over-expression of ICN increased the formation neurosphere-like colonies in the presence of growth factors. These colonies expressed nestin, and these cells could be induced to cell types that expresses specific markers such as neuron, astrocyte, or oligodendrocyte, which is consistent with phenotypes of neural stem cells, suggesting that potential functions of the Notch pathway in the formation of cancer stem cells in human glioma [41]. Fan et al. also found that Notch blockade reduced the CD133-positive cell fraction almost 5-fold and totally abolished the side population. These data suggested that the loss of tumor-forming capacity could be due to the depletion of stem-like cells. Notch signaling levels were higher in the stem-like cell fraction, providing a potential mechanism for their increased sensitivity to the inhibition of this pathway. They also observed that apoptotic rates following Notch blockade were almost 10-fold higher in primitive nestin-positive cells as compared with nestin-negative cells. Stem-like cells in brain tumors thus seems to be selectively vulnerable to agents that will inhibit the Notch pathway [42].

Moreover, Jagged-2, a Notch ligand, was found to be over-expressed in the leukemic stem cells (LSC) samples. DAPT, an inhibitor of gamma-secretase, a protease that is involved in Jagged and Notch signaling, inhibits LSC growth as documented by colony formation assays [43]. Taken together, these results suggested that Notch pathway plays an important role in cancer stem-like cells and thus Notch signaling pathways appears to be a legitimate target for cancer therapy.

5. Notch pathway in multiple cancer and cancer stem cells

Emerging evidence clearly suggest that cancers can grow from cancer stem cells. In cancer stem cells, Notch pathway is believed to be deregulated, leading to uncontrolled self-renewal of cancer stem cells which generate tumor mass. Indeed, Notch gene is abnormally activated in many human malignancies. It has been reported that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of

Notch receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and Large-cell lymphomas and pancreatic cancer [1,2,9,44]. However, in a limited number of tumor types, including human hepatocellular carcinoma, skin and small lung cancer, Notch signaling is anti-proliferative rather than oncogenic [1,2], suggesting that further molecular and mechanistic studies are warranted to ascertain the specific role of Notch family of proteins in cancer stem-like cells. High-level expression of Notch-1 and Jagged-1 was found to be associated with poor prognosis in breast cancer and prostate cancer. The tumor expressing high levels of Jagged-1 protein in patients had a worse outcome than those with tumors expressing low levels [45,46]. Recent reports have shown that Notch-1 expression regulates cell death through both apoptosis and cell cycle pathways in erythroleukemia cells with regulation of c-Jun N-terminal kinase (JNK), Bcl-xL, p21^{cip1}, p27^{kip1}, NF-κB and the retinoblastoma protein Rb [2,47]. The growing body of literature strongly suggests the biological relevance of Notch signaling in cancer cell growth, invasion and metastasis, which further suggest that the inactivation of Notch signaling by novel approach could be useful for cancer therapy. Interestingly, we found that Notch is much more activated in gemcitabine resistant L3.6pl pancreatic cancer cells which showed typical characteristics of EMT phenotype as reported previously [48]. Moreover, we have previously found that a natural product, curcumin is a potent agent in the down-regulation of Notch signaling in pancreatic cancer [49]; however the mechanistic role and the consequence of the down-regulation of Notch signaling in EMT-type cells remains to be elucidated.

Current cancer therapeutic strategies based on tumor regression may target and kill differentiated tumor cells, which constitute the bulk of the tumor, while sparing the rare cancer stem cell population. The cancer stem cell model suggests that the design of new cancer therapeutics may require the targeting and elimination of cancer stem cells. Therefore eradicating cancer stem cells is an important goal in curing cancer, and thus the Notch pathway is considered an attractive target for treatment of cancer because Notch-targeting will not only kill differentiated cancer cells but could also kill cancer stem cells. Moreover reducing Notch activity in cancer stem cells could promote their differentiation, leading to reduce their ability to repopulate the cells in forming tumor mass. These hypotheses must be molecularly and mechanistically tested in multiple tumor system in order to fully appreciate the role of Notch signaling in cancer stem cells. Since Notch signaling is activated via the activity of γ-secretase; therefore inhibitors of γ-secretase viewed as novel target for therapy and, as such, some inhibitors are being tested in Phase I clinical trials, which underscores the importance of Notch signaling for cancer therapy. These results clearly suggest that inactivation of Notch signaling by novel approaches is likely to have a significant impact in cancer therapy. However, it is imperative to design new strategies based upon molecular understanding of the Notch and other signaling pathways that controls the biology of self-renewal and survival capacity of cancer stem-like cells that are reminiscent of EMT phenotype.

Conflict of interest

No.

Acknowledgments

References

[1] L. Miele, Notch signaling, Clin. Cancer Res. 12 (2006) 1074–1079.
[2] L. Miele, H. Miao, B.J. Nickoloff, Notch signaling as a novel cancer therapeutic target, Curr. Cancer Drug Targets 6 (2006) 313–323.
[3] S.K. Mungamuri, X. Yang, A.D. Thor, K. Somasundaram, Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53, Cancer Res. 66 (2006) 4715–4724.
[4] P. Nair, K. Somasundaram, S. Krishna, Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway, J. Virol. 77 (2003) 7106–7112.
[5] T. Nakamura, K. Tsuchiya, M. Watanabe, Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision, J. Gastroenterol. 42 (2007) 705–710.
[6] B.J. Nickoloff, J.Z. Qin, V. Chaturvedi, M.F. Denning, B. Bonish, L. Miele, Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma, Cell Death Differ. 9 (2002) 842–855.
[7] C. Osipo, T.E. Golde, B.A. Osborne, L.A. Miele, Off the beaten pathway: the complex cross talk between Notch and NF-kappaB, Lab. Invest. 88 (2008) 11–17.
[8] A. Rangarajan, R. Syal, S. Selvarajah, O. Chakrabarti, S. Krishna, Activated Notch1 signaling cooperates with papillomavirus oncogenes in transformation and generates resistance to apoptosis on matrix withdrawal through PKB/Akt, Virology 286 (2001) 23–30.
[9] Z. Wang, S. Banerjee, Y. Li, K.M. Rahman, Y. Zhang, F.H. Sarkar, Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{kappa}B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells, Cancer Res. 66 (2006) 2778–2784.
[10] A. Wilson, F. Radtke, Multiple functions of Notch signaling in self-renewing organs and cancer, FEBS Lett. 580 (2006) 2860–2868.
[11] E. Charafe-Jauffret, F. Monville, C. Ginestier, G. Dontu, D. Birnbaum, M.S. Wicha, Cancer stem cells in breast: current opinion and future challenges, Pathobiology 75 (2008) 75–84.
[12] C.D. Peacock, D.N. Watkins, Cancer stem cells and the ontogeny of lung cancer, J. Clin. Oncol. 26 (2008) 2883–2889.
[13] X. Fan, C.G. Eberhart, Medulloblastoma stem cells, J. Clin. Oncol. 26 (2008) 2821–2827.
[14] M. Kakarala, M.S. Wicha, Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy, J. Clin. Oncol. 26 (2008) 2813–2820.
[15] D.H. Scoville, T. Sato, X.C. He, L. Li, Current view: intestinal stem cells and signaling, Gastroenterology 134 (2008) 849–864.
[16] M. Kakarala, M.S. Wicha, Cancer stem cells: implications for cancer treatment and prevention, Cancer J. 13 (2007) 271–275.
[17] B. Ohlstein, T. Kai, E. Decotto, A. Spradling, The stem cell niche: theme and variations, Curr. Opin. Cell Biol. 16 (2004) 693–699.
[18] S.J. Bray, Notch signalling: a simple pathway becomes complex, Nat. Rev. Mol. Cell Biol. 7 (2006) 678–689.
[19] N.M. Joseph, S.J. Morrison, Toward an understanding of the physiological function of Mammalian stem cells, Dev. Cell 9 (2005) 173–183.
[20] B. Keith, M.C. Simon, Hypoxia-inducible factors, stem cells, and cancer, Cell 129 (2007) 465–472.

[21] H. Korkaya, M.S. Wicha, Selective targeting of cancer stem cells: a new concept in cancer therapeutics, BioDrugs 21 (2007) 299–310.

[22] C. Tang, B.T. Ang, S. Pervaiz, Cancer stem cell: target for anti-cancer therapy, FASEB J. 21 (2007) 3777–3785.

[23] B. Ohlstein, A. Spradling, The adult Drosophila posterior midgut is maintained by pluripotent stem cells, Nature 439 (2006) 470–474.

[24] B. Ohlstein, A. Spradling, Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling, Science 315 (2007) 988–992.

[25] E.J. Ward, H.R. Shcherbata, S.H. Reynolds, K.A. Fischer, S.D. Hatfield, H. Ruohola-Baker, Stem cells signal to the niche through the Notch pathway in the Drosophila ovary, Curr. Biol. 16 (2006) 2352–2358.

[26] M.V. Gustafsson, X. Zheng, T. Pereira, et al, Hypoxia requires notch signaling to maintain the undifferentiated cell state, Dev. Cell 9 (2005) 617–628.

[27] S. Fre, M. Huyghe, P. Mourikis, S. Robine, D. Louvard, S. rtavanis-Tsakonas, Notch signals control the fate of immature progenitor cells in the intestine, Nature 435 (2005) 964–968.

[28] J. Styczynski, T. Drewa, Leukemic stem cells: from metabolic pathways and signaling to a new concept of drug resistance targeting, Acta Biochim. Pol. 54 (2007) 717–726.

[29] M. Katoh, Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis, Stem Cell Rev. 3 (2007) 30–38.

[30] G. Dontu, K.W. Jackson, E. McNicholas, M.J. Kawamura, W.M. Abdallah, M.S. Wicha, Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells, Breast Cancer Res. 6 (2004) R605–R615.

[31] T.M. Phillips, K. Kim, E. Vlashi, W.H. McBride, F. Pajonk, Effects of recombinant erythropoietin on breast cancer-initiating cells, Neoplasia 9 (2007) 1122–1129.

[32] G. Farnie, R.B. Clarke, Mammary stem cells and breast cancer—role of Notch signalling, Stem Cell Rev. 3 (2007) 169–175.

[33] S.K. Singh, I.D. Clarke, M. Terasaki, et al, Identification of a cancer stem cell in human brain tumors, Cancer Res. 63 (2003) 5821–5828.

[34] P. Dell'albani, Stem cell markers in gliomas, Neurochem. Res. (2008), in press [Epub ahead of print].

[35] L. Patrawala, T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool, D.G. Tang, Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and A, Cancer Res. 65 (2005) 6207–6219.

[36] Y. Nakamura, S. Sakakibara, T. Miyata, et al, The bHLH gene hes1 as a repressor of the neuronal commitment of CNS stem cells, J. Neurosci. 20 (2000) 283–293.

[37] S. Hitoshi, R.M. Seaberg, C. Koscik, et al, Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling, Genes Dev. 18 (2004) 1806–1811.

[38] S. Hitoshi, T. Alexson, V. Tropepe, et al, Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells, Genes Dev. 16 (2002) 846–858.

[39] K. Mellodew, R. Suhr, D.A. Uwanogho, et al, Nestin expression is lost in a neural stem cell line through a mechanism involving the proteasome and Notch signalling, Brain Res. Dev. Brain Res. 151 (2004) 13–23.

[40] A.H. Shih, E.C. Holland, Notch signaling enhances nestin expression in gliomas, Neoplasia 8 (2006) 1072–1082.

[41] X.P. Zhang, G. Zheng, L. Zou, et al, Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells, Mol. Cell. Biochem. 307 (2008) 101–108.

[42] X. Fan, W. Matsui, L. Khaki, et al, Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors, Cancer Res. 66 (2006) 7445–7452.

[43] H. Gal, N. Amariglio, L. Trakhtenbrot, et al, Gene expression profiles of AML derived stem cells; similarity to hematopoietic stem cells, Leukemia 20 (2006) 2147–2154.

[44] Z. Wang, Y. Zhang, Y. Li, S. Banerjee, J. Liao, F.H. Sarkar, Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells, Mol. Cancer Ther. 5 (2006) 483–493.

[45] B.C. Dickson, A.M. Mulligan, H. Zhang, et al, High-level JAG1 mRNA and protein predict poor outcome in breast cancer, Mod. Pathol. 20 (2007) 685–693.

[46] S. Santagata, F. Demichelis, A. Riva, et al, JAGGED1 expression is associated with prostate cancer metastasis and recurrence, Cancer Res. 64 (2004) 6854–6857.

[47] M.S. Jang, H. Miao, N. Carlesso, et al, Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways, J. Cell Physiol. 199 (2004) 418–433.

[48] A.N. Shah, J.M. Summy, J. Zhang, S.I. Park, N.U. Parikh, G.E. Gallick, Development and characterization of gemcitabine-resistant pancreatic tumor cells, Ann. Surg. Oncol. 14 (2007) 3629–3637.

[49] Z. Wang, Y. Zhang, S. Banerjee, Y. Li, F.H. Sarkar, Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells, Cancer 106 (2006) 2503–2513.